Predictive biomarkers in diffuse gliomas and non-small cell lung cancer

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I **Tuononen K***, Tynninen O*, Sarhadi VK, Tyybäkinoja A, Lindlöf M, Antikainen M, Näpänkangas J, Hirvonen A, Mäenpää H, Paetau A, Knuutila S. The hypermethylation of the O^6-methylguanine-DNA methyltransferase gene promoter in gliomas – correlation with array comparative genome hybridization results and IDH1 mutation. *Genes Chromosomes Cancer* 2012;51(1):20-9.

II **Tuononen K**, Sarhadi VK, Wirtanen A, Ronty M, Salmenkivi K, Knuuttila A, Remes S, Telaranta-Keerie AI, Bloor S, Ellonen P, Knuutila S. Targeted resequencing reveals ALK fusions in non-small cell lung carcinomas detected by FISH, immunohistochemistry, and real-time RT-PCR: a comparison of four methods. *Biomed Res Int* 2013:757490.

III **Tuononen K**, Mäki-Nevala S, Sarhadi VK, Wirtanen A, Rönty M, Salmenkivi K, Andrews JM, Telaranta-Keerie AI, Hannula S, Lagström S, Ellonen P, Knuuttila A, Knuutila S. Comparison of targeted next-generation sequencing (NGS) and real-time PCR in the detection of EGFR, KRAS, and BRAF mutations on formalin-fixed, paraffin-embedded tumor material of non-small cell lung carcinoma – superiority of NGS. *Genes Chromosomes Cancer* 2013;52(5):503-11.

IV **Tuononen K**, Kero M, Mäki-Nevala S, Sarhadi V, Tikkanen M, Wirtanen T, Rönty M, Knuuttila A, Knuutila S. ALK fusion and its association with other driver gene mutations in Finnish non-small cell lung cancer patients. *Genes Chromosomes Cancer* 2014;53(11):895-901.

*These authors contributed equally to the study.

ABBREVIATIONS

3´UTR	three prime untranslated region
5´UTR	five prime untranslated region
AKT1	v-akt murine thymoma viral oncogene homolog 1
ALK	anaplastic lymphoma receptor tyrosine kinase
ARMS	Amplification Refractory Mutation System
array CGH	array comparative genomic hybridization
ATP	adenosine triphosphate
BRAF	v-raf murine sarcoma viral oncogene homolog B
CDKN2A	cyclin-dependent kinase inhibitor 2A
CI	confidence interval
COSMIC	Catalogue Of Somatic Mutations In Cancer
CTNNB1	catenin (cadherin-associated protein), beta 1, 88 kDa
DDR2	discoidin domain receptor tyrosine kinase 2
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EGFR	epidermal growth factor receptor
EML4	echinoderm microtubule-associated protein-like 4
FDA	Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
FGFR1	fibroblast growth factor receptor 1
FIMM	Institute for Molecular Medicine Finland
FISH	fluorescence in situ hybridization
HDAC	histone deacetylase
HER2 (ERBB2)	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
HR	hazard ratio
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble
IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial
IGV	Integrative Genomics Viewer
IHC	immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene homolog
LOH	loss of heterozygosity
MEK1 (MAP2K1)	mitogen-activated protein kinase kinase 1
MET	met proto-oncogene
MGMT	O^6-methylguanine-DNA methyltransferase
miRNA	micro-RNA
mRNA	messenger ribonucleic acid
MS-HRM	methylation-sensitive high-resolution melting

MS-MLPA	methylation-specific multiplex ligation-dependent probe amplification
MSP	methylation-specific PCR
NGS	next-generation sequencing
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
NSCLC	non-small cell lung cancer
NTRK1	neurotrophic tyrosine kinase, receptor, type 1
OS	overall survival
PCR	polymerase chain reaction
PCV	procarbazine, lomustine (CCNU), and vincristine
PE	paired-end
PFS	progression-free survival
PGM	Personal Genome Machine
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PPi	pyrophosphate
PTEN	phosphatase and tensin homolog
RET	ret proto-oncogene
RNA	ribonucleic acid
ROS1	ROS proto-oncogene 1, receptor tyrosine kinase
RT-PCR	reverse transcription polymerase chain reaction
SCLC	small cell lung cancer
SNV	single-nucleotide variant
TKI	tyrosine kinase inhibitor
TNM	tumor, node, metastasis
TP53	tumor protein p53
VCP	variant-calling pipeline
VEGF	vascular endothelial growth factor
WHO	World Health Organization

Gene symbols are marked in the text in *italics* and according to the guidelines of the Human Genome Organization nomenclature committee (HGNC). Full names for genes not listed here can be found at http://www.genenames.org/.

ABSTRACT

The presence of certain cancer-related genetic and epigenetic alterations in the tumor affect patients´ response to specific cancer therapies. The accurate screening of these predictive biomarkers in molecular diagnostics is important since it enables the tailoring of an optimal treatment based on molecular characteristics of the tumor. Depending on the type of gene alteration, a wide variety of methods could be applied in biomarker testing. Among the novel methods is next-generation sequencing (NGS) technology, enabling simultaneous detection of multiple alterations. The aim of this thesis was to analyze predictive or potentially predictive genetic and epigenetic alterations of diffuse gliomas and non-small cell lung cancer (NSCLC), and to evaluate the feasibility of pyrosequencing and targeted NGS in the detection of these alterations in formalin-fixed paraffin-embedded (FFPE) tumor tissue specimens.

In Study I, we assessed the genetic and epigenetic profile of diffuse gliomas by applying methylation-specific pyrosequencing to detect *MGMT* promoter hypermethylation, array comparative genomic hybridization to detect chromosomal copy number alterations, and immunohistochemistry (IHC) to detect *IDH1* mutation status. *MGMT* hypermethylation, *IDH1* mutations, and losses of chromosome arms 1p and 19q were typical changes in oligodendroglial tumors (grades II-III), whereas losses of 9p and 10q were frequently seen in glioblastomas (grade IV). Furthermore, we detected significant associations of 1) *MGMT* hypermethylation with *IDH1* mutations and loss of 19q, 2) unmethylated *MGMT* with losses of 9p and 10q and gain of 7p, 3) *IDH1* mutations with *MGMT* hypermethylation, 1p loss, and combined loss of 1p/19q, and 4) non-mutated *IDH1* with losses of 10q. Pyrosequencing proved to be a feasible method for determination of *MGMT* methylation status in FFPE sample material.

In Studies II and III, we compared targeted NGS with fluorescence in situ hybridization, IHC, and real-time reverse-transcription PCR in the detection of *ALK* fusion (Study II), and with real-time PCR in the detection of *EGFR*, *KRAS*, and *BRAF* mutations (Study III). All analyses were successfully performed on all FFPE samples. A good concordance was observed between the results obtained by different methods, and targeted NGS also proved to be advantageous in the identification of novel and rare variants with a potential predictive value.

In Study IV, we determined the frequency of *ALK* fusion in 469 Finnish NSCLC patients, and the association of *ALK* fusion with clinicopathological characteristics and with the presence of mutations in 22 other driver genes. We detected *ALK* fusion at a frequency of 2.3%, suggesting that it is a relatively rare alteration in Finnish NSCLC patients. The presence of *ALK* fusion was significantly linked to younger age and never-/ex-light smoking history. Although most of the ALK-positive tumors had adenocarcinoma

histology, also ALK-positive large cell carcinomas were detected. Characterization of ALK-positive cases by targeted NGS showed a coexistence of *ALK* fusion with mutations in *MET*, *TP53*, *CTNNB1*, and *PIK3CA*, but the value of these co-occurrences requires further examination.

In conclusion, our studies indicate that certain genetic and epigenetic alterations occur together, and the simultaneous screening of multiple alterations may thus allow one to obtain a more comprehensive picture of the molecular background of the tumor, which could facilitate prediction of tumor behavior, prognosis, and treatment response. Our results show the feasibility of pyrosequencing and targeted NGS in FFPE tumor tissue material and also the advantages of targeted NGS over other commonly used methods in the detection of gene rearrangements and mutations, particularly the ability to simultaneously identify multiple alterations.

TIIVISTELMÄ

Tiettyjen syöpään liityvien geneettisten ja epigeneettisten muutosten esiintyminen kasvaimissa vaikuttaa potilaan vasteeseen syöpähoidoille. Näiden hoitovastetta ennustavien, prediktiviisten biomarkkereiden tarkka seulonta molekyylidiagnostiikassa on tärkeää, sillä se mahdollistaa optimaalisen hoidon räätälöinnin kasvaimen molekulaaristen ominaisuuksien perusteella. Geenimuutoksesta riippuen biomarkkereiden testauksessa voidaan hyödyntää useita eri menetelmiä, joista uusimpien joukossa ovat lukuisten markkereiden samanaikaisen tutkimisen mahdollistavat uuden sukupolven sekvensointimenetelmät. Tämän väitöskirjatutkimuksen tavoitteena oli analysoida hoitovastetta ennustavia geneettisiä ja epigeneettisiä muutoksia glioomissa ja ei-pienisoluisessa keuhkosyövässä. Lisäksi työssä arvioitiin pyrosekvensoinnin ja kohdennetun uuden sukupolven sekvensoinnin soveltuvuutta näiden muutosten tunnistamiseen formaliiniin fiksoiduista ja parafiiniin valetuista (FFPE) kasvainkudosnäytteistä.

Ensimmäisessä osatyössä tutkimme 51 glioomanäytettä määrittämällä niistä *MGMT*-geenin säätelyalueen metyloitumisasteen pyrosekvensoinnilla, kromosomien kopiolukumäärämuutokset mikrosirupohjaisella vertailevalla genomisella hybridisaatiolla ja *IDH1*-geenin mutaatiostatuksen immunohistokemiallisella värjäyksellä. Tutkimuksessa havaitsimme vaihtelua tutkittujen biomarkkerien esiintymisessä eri glioomatyyppien välillä: *MGMT*-geenin metylaatio, *IDH1*-geenin mutaatiot sekä kromosomien 1p ja 19q puutokset olivat tyypillisiä muutoksia oligodendrogliaalisissa kasvaimissa (gradus II-III), kun taas kromosomien 9p ja 10q puutoksia nähtiin useammin glioblastoomissa (gradus IV). Lisäksi osoitimme, että 1) metyloitunut *MGMT*-geeni esiintyy usein yhdessä *IDH1*-geenin mutaation ja kromosomin 19q puutoksen kanssa, 2) metyloitumaton *MGMT*-geeni kromosomien 9p ja 10q puutosten sekä kromosomin 7q monistuman kanssa 3) mutatoitunut *IDH1*-geeni metyloituneen *MGMT*-geenin sekä kromosomien 1p ja 1p/19q puutosten kanssa, sekä 4) mutatoitumaton *IDH1*-geeni kromosomin 10q puutoksen kanssa. Pyrosekvensointi osoittautui soveltuvan hyvin *MGMT*-geenin metyloitumisasteen määrittämiseen FFPE-materiaalista.

Osatöissä II ja III vertasimme kohdennettua uuden sukupolven sekvensointia fluoresenssi in situ hybridisaatioon, immunohistokemialliseen värjäykseen ja käänteistranskriptaasi-PCR-menetelmään *ALK*-geenin fuusioiden tunnistamisessa (osatyö II), sekä PCR-menetelmään *EGFR*-, *KRAS*-, ja *BRAF*-geenimutaatioiden tunnistamisessa (osatyö III). Tuloksemme osoittavat, että käytetyt menetelmät soveltuvat FFPE-näytteille ja eri menetelmillä saadut tulokset vastaavat hyvin toisiaan. Lisäksi kohdennettu uuden

sukupolven sekvensointi osoittautui hyödylliseksi tunnistettaessa uusia ja harvinaisia geenimuutoksia, joilla saattaa olla prediktiivistä arvoa.

Osatyössä IV määritimme *ALK*-geenin fuusioiden esiintymisen suomalaisessa potilasaineistossa, joka koostui 469 ei-pienisoluista keuhkosyöpää sairastavasta potilaasta. Lisäksi tutkimme lähemmin *ALK*-geenin fuusiota kantavien potilaiden ominaisuuksia sekä fuusiogeenin esiintymistä 22 muun geenin mutaatioiden kanssa. Immunohistokemiallisen värjäyksen avulla määritimme *ALK*-geenin fuusioiden esiintyvän suhteellisen alhaisella frekvenssillä (2,3%) suomalaisissa keuhkosyöpäpotilaissa. ALK-positiiviset potilaat olivat merkittävästi nuorempia ja useammin täysin tupakoimattomia tai entisiä vähän tupakoivia. Vaikka suurin osa ALK-positiivisista kasvaimista oli adenokarsinoomia, *ALK*-geenin fuusioita havaittiin myös suurisoluisissa karsinoomissa. Kohdennetun uuden sukupolven sekvensoinnin avulla havaittiin *ALK*-geenin fuusioita esiintyvän yhdessä geeneissä *MET*, *TP53*, *CTNNB1*, ja *PIK3CA* esiintyvien mutaatioiden kanssa, mutta lisätutkimuksia tarvitaan selvittämään näiden geenien yhteisesiintymisten merkitys.

Tutkimuksemme osoittavat, että jotkin geneettiset ja epigeneettiset muutokset esiintyvät yhdessä. Näin ollen useita muutoksia samanaikaisesti tutkimalla voidaan saada kokonaisvaltaisempi kuva kasvaimen molekulaarisesta taustasta, mikä voi edesauttaa kasvaimen käytöksen, ennusteen ja hoitovasteen arvioimisessa. Lisäksi tuloksemme osoittavat pyrosekvensoinnin ja uuden sukupolven sekvensoinnin soveltuvuuden FFPE-kasvainkudosnäytteille, sekä kohdennetun uuden sukupolven sekvensoinnin tuoman hyödyn geenifuusioiden ja -mutaatioiden tunnistamisessa muihin käytettyihin menetelmiin verrattuna, kuten mahdollisuuden selvittää samanaikaisesti useita geenimuutoksia.

INTRODUCTION

Various genetic and epigenetic alterations contribute to cancer development, including numerical and structural chromosome aberrations, small DNA alterations, DNA methylation, histone modifications, and deregulated expression of small non-coding micro-RNAs (miRNAs). The presence of some of these genetic and epigenetic alterations may help to predict the response of the cancer patient to a specific treatment, thus serving as a predictive biomarker. Some alterations confer sensitivity to treatment, while others may be associated with resistance. An increasing number of therapeutic agents targeting specific cancer-related alterations are developed and investigated, the use of which may improve the survival of patients harboring the relevant alterations.

Accurate screening for predictive biomarkers in routine molecular diagnostics is important to direct treatment to those who will likely benefit from it. Various different technologies exist for identification of different predictive biomarkers. Methylation-specific pyrosequencing is one of the methods applicable for detection of methylation. It provides accurate quantitative information of the degree of methylation at individual CpG sites. The development of an increasing number of novel targeted treatments indicates the importance of applying methods for analysis of predictive biomarkers that enable the simultaneous identification of multiple alterations. Targeted next-generation sequencing (NGS) is a promising method for this purpose since it provides time- and cost-efficient assessment of numerous alterations.

Gliomas are the most frequent malignant brain tumors arising from the supportive glial cells of the brain. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer originating from epithelial cells of the lung. High-grade diffuse gliomas and advanced stage NSCLCs are associated with poor survival, which could be improved by the use of targeted molecular therapies.

This thesis focuses on the analyses of predictive biomarkers in adult diffuse gliomas and NSCLCs. The use of pyrosequencing in the detection of gene promoter methylation, and targeted NGS in the detection of gene rearrangements and mutations is evaluated in formalin-fixed paraffin-embedded tumor tissue specimens.

REVIEW OF THE LITERATURE

1. Genetic and epigenetic alterations in cancer

Cancer is a common complex disease arising from the malignant transformation and uncontrolled growth of a cell due to accumulation of genetic and epigenetic modifications affecting genome stability. Two to eight alterations are thought to suffice for development of the majority of cancers, with each alteration directly or indirectly increasing the growth advantage of the cell (Vogelstein et al., 2013). The two types of genes generally involved in tumor development are proto-oncogenes, which code for proteins participating in cell proliferation, division, or differentiation, and tumor suppressor genes, which code for proteins related to inhibition of cell proliferation. Also, DNA repair genes are considered to be tumor suppressor genes. Activating alterations in proto-oncogenes convert them into oncogenes, which promote cell proliferation, whereas inactivating alterations in tumor suppressor genes lead to loss of control of cell proliferation. During multistep tumorigenesis cancer cells are suggested to acquire the following hallmarks: 1) self-sufficiency in growth signals, 2) insensitivity to antigrowth signals, 3) evasion of apoptosis, 4) unlimited replicative potential, 5) sustained angiogenesis, 6) tissue invasion and metastasis, 7) reprogramming of energy metabolism, and 8) evasion of immune destruction (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The risk of developing a cancer depends not only on the individual´s genetic background, and inheritance of cancer-predisposing genetic variants, but also on the exposure to environmental and life-style factors such as tobacco smoking, alcohol, obesity, infectious agents (viruses, bacteria, parasites), environmental pollution, radiation, and carcinogens related to food and cooking (reviewed by Anand et al., 2008), which may induce genetic or epigenetic alterations. Interactions between genetic and environmental factors further contribute to cancer risk.

1.1 Genetic alterations in cancer

Various changes in the genome, including small sequence alterations, as well as larger structural and numerical chromosome aberrations are frequently seen in cancer, single-base substitutions being the most prevalent alteration type in tumors (Vogelstein et al., 2013). These changes may alter the normal function of the protein products of proto-oncogenes and tumor suppressor genes, thus contributing to cancer development. The proteins encoded by oncogenes and their associated signaling pathways serve as targets for many therapeutic agents, including small-molecule inhibitors and monoclonal antibodies, which block oncogenic processes such as aberrant signaling (reviewed by Ciavarella et al., 2010). To date, many drugs designed to inhibit these targets have

received approval by the U.S. Food and Drug Administration (FDA) for treatment of various cancers (Abramson, 2014).

1.1.1 Small DNA sequence alterations

Small changes in DNA sequence, including substitutions, insertions, and deletions of one or a few nucleotides, affect the codon composition and may further alter the amino acid and protein structure (Figure 1). The substitutions of one base by another, known as single-nucleotide variants (SNVs) or point mutations, can lead to an alteration in the sequence that 1) does not change the amino acid, and thus, has no effect on the protein (silent mutation), 2) changes the amino acid, with potentially harmful consequences on the protein (missense mutation), or 3) causes an early stop codon, resulting in a shortened protein product (nonsense mutation). The changes affecting the amino acid sequence are also called non-synonymous alterations, whereas synonymous alterations have no effect on the amino acid sequence. Insertions and deletions of nucleotides can lead to alterations of the reading frame (frameshift mutation), and insertions can also affect the splicing of the introns (splice site mutation). When harmful sequence alterations occur in the coding regions of cancer-related genes, they may lead to activation of proto-oncogenes or inactivation of tumor suppressor genes. Point mutations and small deletions in the epidermal growth factor receptor (*EGFR*) gene, leading to its oncogenic activation, provide an example of small sequence alterations frequently detected in lung cancer (Pao et al., 2004).

1.1.2 Structural and numerical chromosome alterations

Structural chromosome alterations include deletions, duplications, inversions, substitutions, and translocations of chromosome arms or fragments of chromosomes (Figure 1). Deletions of chromosome parts lead to losses of genetic material, and duplications to gains of genetic material. Amplifications represent a higher increase in the number of copies of chromosome regions. Inclusion of proto-oncogenes in the duplicated or amplified chromosome regions may result in an overexpression of the encoded proteins, whereas deletion of chromosome regions containing tumor suppressor genes may cause a decrease in the expression of the encoded proteins. Translocations, inversions, and substitutions are rearrangements of DNA sequences between different chromosomes or within a single chromosome, which may result in a fusion of genes that are normally separated. The gene fusion could lead to a disruption of a gene at the breakpoint or generate a fused gene carrying a function that can promote tumorigenesis, especially when proto-oncogenes or tumor suppressor genes are involved at the

breakpoint. Structural chromosome alterations are classified as balanced when no genetic material is gained or lost, or unbalanced when gain or loss of genetic material is detected.

Numerical chromosome alterations consist of gains and losses of whole chromosomes (aneuploidy). Although the actual role of aneuploid chromosomes in cancer is mostly unknown, it may be related to, for example, amplification of oncogenes, loss of tumor suppressor genes, or decrease in genome stability (reviewed by Gordon et al., 2012). Examples of chromosome alterations involved in cancer include deletion of chromosome 10q and *EGFR* gene amplification in glioblastomas (Ohgaki et al., 2004), as well as an oncogenic activation of anaplastic lymphoma receptor tyrosine kinase (*ALK*) gene by its fusion with echinoderm microtubule-associated protein-like 4 (*EML4*) gene in NSCLC (Soda et al., 2007).

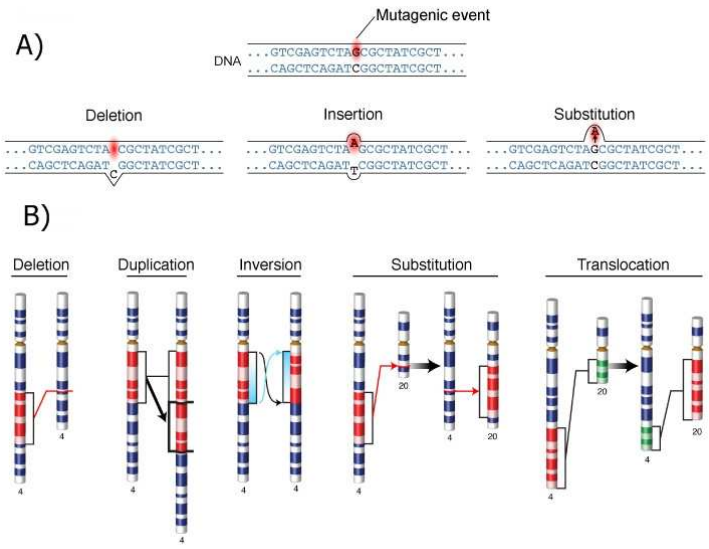

Figure 1. Common genetic alterations in cancer. A) Small DNA sequence alterations, including deletion, insertion, and substitution of one or a few base pairs. B) Structural chromosome alterations, including deletion, duplication, inversion, substitution, and translocation of chromosome regions. Image courtesy of National Human Genome Research Institute.

1.2 Epigenetic alterations in cancer

Epigenetic alterations, including DNA methylation, histone modifications, and deregulated expression of small non-coding miRNAs, are important contributors of

tumorigenesis (Figure 2). These potentially reversible alterations affect gene expression without changing the DNA sequence.

1.2.1 DNA methylation

DNA methylation is an epigenetic event, in which a methyl group is added to cytosine of a CpG dinucleotide by enzymes known as DNA methyltransferases (DNMTs). Locally clustered CpG dinucleotides, called CpG islands, are predominantly found at the 5´end of many genes, and they are usually unmethylated in normal cells, although methylated promoter CpG islands are seen in some processes during normal development such as in genomic imprinting and X chromosome inactivation (reviewed by Bird, 2002). The majority of CpG sites located elsewhere in the genome, such as in the repetitive sequences, are generally highly methylated (Bird, 2002). The alterations in the methylation status of genes and genome have been frequently detected in tumors including, for example, the inactivation of tumor suppressor genes by hypermethylation of the CpG islands in their promoter regions, as well as hypomethylation of some normally methylated genomic regions, which might lead to genomic instability, reactivation of transposable elements, loss of imprinting, and activation of silent genes, thus contributing to tumorigenesis (reviewed by Esteller, 2008). An example of aberrant DNA methylation frequently found in specific types of cancer, such as gliomas, includes the hypermethylation of DNA repair gene O^6-methylguanine-DNA methyltransferase (*MGMT*), leading to silencing of the gene (Esteller et al., 2000).

Inhibitors of DNMTs, the increased expression of which is seen in many cancers, have been developed as drugs for cancer therapy (reviewed by Subramaniam et al., 2014). By inhibiting DNMTs, tumorigenicity could be reduced, as the expression of tumor suppressor genes is increased. To date, two DNMT inhibitors, azacytidine and decitabine, have obtained FDA approval for treatment of myelodysplastic syndromes (reviewed by Gnyszka et al., 2013).

1.2.2 Histone modifications

Histone proteins (H2A, H2B, H3, and H4, two copies of each), together with DNA wrapped around them, form the basic structure of chromatin, called the nucleosome. The protruding N-terminal tails of histones provide sites for posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, adenosine diphosphate ribosylation, deimination, and proline isomerization (reviewed by Kouzarides, 2007). Depending on the type of modifications and residues of the histone tails modified, the effects on chromatin structure, and further on gene transcription, DNA

repair, and DNA replication are different; some of them lead to loose, transcriptionally active euchromatin, whereas others lead to more condensed, transcriptionally inactive heterochromatin (Kouzarides 2007). Aberrations in histone modifications and histone-modifying complexes are associated with cancer development (reviewed by Sawan and Herceg, 2010). For example, altered histone H4 modifications, including hyperacetylation of H4 K5 and H4 K8 and hypoacetylation of H4 K12 and H4 K16, have been identified in NSCLC cells (Van Den Broeck et al., 2008).

Inhibitors for histone deacetylases (HDACs), the enzymes regulating gene expression by removing acetyl groups from histones, have been developed as anticancer agents (reviewed by West and Johnstone, 2014). The functions of HDAC inhibitors include induction of tumor cell apoptosis, growth arrest, senescence, differentiation, and immunogenicity, and inhibition of angiogenesis. To date, three HDAC inhibitors, vorinostat, romidepsin, and belinostat, have obtained FDA approval for treatment of cutaneous and peripheral T-cell lymphoma (Abramson, 2014).

1.2.3 Small non-coding miRNAs

MiRNAs are small non-coding RNAs, approximately 22 nucleotides in length, that bind sequence-specifically to messenger RNAs (mRNAs), and by mRNA degradation or inhibition of translation, miRNAs alter the gene expression of the target gene (reviewed by Bartel, 2004). MiRNAs are important in regulation of many processes in normal cells, including proliferation, apoptosis, and differentiation, but their altered expression (under- or overexpression) is associated with tumor development, and numerous deregulated miRNA expression patterns have been detected in various cancers (reviewed by Mirnezami et al., 2009). An example of involvement of miRNA in lung cancer is a reduced expression of *let-7*, with a possible role in increasing expression of the *RAS* oncogene (Takamizawa et al., 2004; Johnson et al., 2005).

Small-molecule inhibitors of specific miRNAs, antisense oligonucleotides, locked nucleic acids, antagomirs, miRNA sponges, small interfering RNAs, and short hairpin RNAs are examples of treatment approaches for inhibition of oncogenic miRNAs, which are overexpressed in cancer and target tumor suppressor proteins (reviewed by Monroig et al., 2015). Small molecules affecting the RNA interference pathway provide an example of treatment strategies for recovery of tumor suppressor miRNAs, which are underexpressed in cancer and target oncogenes. To date, none of the studied agents has gained FDA approval as miRNA-targeting agents. Control of off-target effects and improved delivery are among the future challenges of miRNA therapies in cancer.

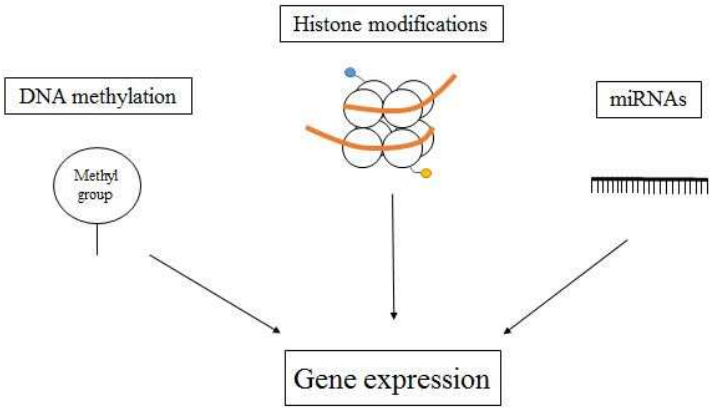

Figure 2. Epigenetic alterations affecting gene expression.

1.3 Predictive biomarkers for targeted therapies

Some cancer-related genetic and epigenetic alterations driving tumorigenesis ("driver mutations") are considered to be predictive biomarkers because their presence predicts the patients´ response to a specific therapy. Screening for predictive biomarkers enables tailoring of the cancer treatment based on the tumor´s genetic and epigenetic makeup; often expensive and possibly toxic therapies could specifically be prescribed for patients likely to benefit from the treatment. Conversely, treatment could be withheld from those patients unlikely to benefit or predicted to exhibit adverse side-effects. Some alterations may contribute to primary resistance to targeted therapy, and even those tumors with sensitizing alterations and a primary benefit from the therapy might sooner or later acquire resistance to it (reviewed by Garraway and Jänne, 2012). Examples of mechanisms leading to resistance include reactivation of the target due to secondary alterations and activation of upstream or downstream effectors or bypass oncoproteins of the signaling pathways involved in tumorigenesis.

Currently, many molecular targeting drugs, including small-molecule compounds and monoclonal antibodies, have been approved for cancer treatment and many more are undergoing clinical and preclinical studies (Abramson, 2014). These agents act on specific molecules with alterations involved in tumorigenesis. While small-molecule compounds are able to pass through the cell membrane and target cytoplasmic molecules

and domains, monoclonal antibodies can target molecules outside the cell or on the cell surface (reviewed by Imai and Takaoka, 2006). As our understanding of cancer initiation, progression, and metastasis continues to broaden and novel candidates for targeted therapies are identified, the number of targeted drugs is expected to increase. Development of targeted therapies is important because they may improve the outcome of cancer patients.

2. Gliomas

2.1 Epidemiology

Tumors in the brain and central nervous system were estimated to account for 1.8% (256 000) of new cancer cases and 2.1% (142 000) of cancer deaths worldwide in 2012 (Ferlay et al., 2015). In Finland, tumors in the brain and central nervous system comprise 2.6% of all cancers and 3.1% of total cancer deaths in males, while the corresponding figures in females are 4.0% and 3.6%, (NORDCAN, Association of Nordic Cancer Registries, 2013a). This thesis focuses on gliomas, which arise from the supportive glial cells of the brain and account for around 30% of all central nervous system and brain tumors and 80% of malignant brain tumors (Goodenberger and Jenkins, 2012). In addition to heritable risk variants, other factors with a proposed link to gliomas include, for example, ionizing radiation associated with increased risk and allergic conditions associated with reduced risk of gliomas (reviewed by Ostrom et al., 2014). Also, some monogenic Mendelian syndromes, such as neurofibromatosis 1, Li-Fraumeni syndrome, tuberous sclerosis, and Lynch syndrome, predispose to gliomagenesis, but only a small proportion of all glioma cases is explained by these syndromes.

2.2 Histopathology and grading of gliomas

Based on cellular morphology, the World Health Organization (WHO) classification divides gliomas into three major subtypes: astrocytomas, oligodendrogliomas, and a mixture of these two cell types, oligoastrocytomas (Louis et al., 2007a). Both oligondedrogliomas and oligoastrocytomas are also called oligodendroglial tumors. The histological WHO grading system provides information on the biological aspects of the tumors, aiding prognosis and prediction of treatment response. WHO grade I tumors are benign, discrete, and curable by surgical removal, whereas WHO grade II-IV diffuse gliomas infiltrate into the surrounding brain tissue, thus preventing complete surgical removal and cure (Claes et al., 2007; Louis et al., 2007a). Grade II tumors show increased cellularity, grade III tumors also show increased anaplasia and mitotic figures, and grade IV tumors show vascular proliferation and necrosis in addition to the aforementioned features (Louis et al., 2007a). Grade IV astrocytoma, glioblastoma, is the most frequently occurring and most malignant glioma subtype. It is further subdivided into primary glioblastomas (95% of cases), arising without evidence of pre-existing lower grade gliomas, and secondary glioblastomas (5% of cases), developing from lower grade gliomas (Ohgaki et al., 2004; Louis et al., 2007a). Primary and secondary glioblastomas can not be distinguished by histopathology, but they exhibit genetic and epigenetic differences, and patients with secondary glioblastomas are typically younger at diagnosis (Ohgaki and Kleihues, 2007).

Certain molecular alterations are frequently seen in specific glioma subtypes and grades, and thus, they may further aid in the classification of tumors. Examples of these alterations include codeletion of 1p/19q in oligodendroglial tumors (Reifenberger et al., 1994; Bigner et al., 1999; Smith et al., 2000; Okamoto et al., 2004; Miller et al., 2006; Jeuken et al., 2011), *IDH1* mutation in diffuse grade II-III gliomas and secondary glioblastomas (Balss et al., 2008; Hartmann et al., 2009; Watanabe et al., 2009; Yan et al., 2009), and loss of heterozygosity (LOH) of chromosome 10q, *EGFR* amplification, *TP53* mutations, $p16^{INK4a}$ (*CDKN2A*) deletions, and *PTEN* mutations in glioblastomas (Ohgaki et al., 2004). In addition to contributing to the pathogenesis of gliomas, many of these molecular alterations have prognostic significance for prediction of outcome of patients (reviewed by Haynes et al., 2014). For example, codeletion of 1p/19q and *IDH1* mutations have been associated with favorable prognosis, whereas LOH 10q and *PTEN* mutations have been linked to poor prognosis.

Many factors, such as WHO grade, tumor location, age of the patient, performance status, and presence of specific molecular alterations, contribute to the outcome and treatment response of glioma patients (Louis et al., 2007b). Population-based studies have shown the following 5-year survival rates (mean of the studies) for different glioma subtypes and grades: 68.5% in oligodendrogliomas (grade II), 50% in oligoastrocytomas, 41.9% in astrocytomas (grade II), 34.4% in anaplastic oligodendrogliomas (grade III), 19.8% in anaplastic astrocytomas (grade III), and 3.4% in glioblastomas (grade IV) (reviewed by Ostrom et al., 2014). Despite the relatively good survival from slowly growing low-grade gliomas, they eventually will progress to higher-grade gliomas (Riemenschneider et al., 2010).

2.3 Predictive biomarkers in adult diffuse gliomas

2.3.1 Codeletion of chromosomes 1p and 19q

Combined loss of whole chromosome arms 1p and 19q, potentially caused by an unbalanced translocation between the arms early in tumorigenesis (Reifenberger et al., 1994; Jenkins et al., 2006), is a frequent change in oligodendroglial tumors, reported in 44-89% of oligodendrogliomas (Reifenberger et al., 1994; Bigner et al., 1999; Smith et al., 2000; Okamoto et al., 2004; Miller et al., 2006; Jeuken et al., 2011) and in 19-38% of oligoastrocytomas (Bigner et al., 1999; Smith et al., 2000; Miller et al., 2006; Jeuken et al., 2011). In astrocytomas/glioblastomas, the codeletion of 1p/19q is a rare event (Smith et al., 2000; Miller et al., 2006; Jeuken et al., 2011). Although the tumor suppressor genes involved in the 1p/19q loss have not been unequivocally identified, some candidate genes have been discovered within the lost chromosome arms, including genes coding for capicua transcriptional repressor (CIC) located at 19q13.2 and far upstream element

(FUSE) binding protein 1 (FUBP1) located at 1p31.1 (Bettegowda et al., 2011). In patients diagnosed with anaplastic oligodendroglial tumors, the codeletion of 1p/19q has been associated with a better survival when the patients are treated with radiotherapy and combination chemotherapy of alkylating agents procarbazine and lomustine (CCNU) together with microtubule inhibitor vincristine (PCV) compared with radiotherapy alone (Cairncross et al., 2013; van den Bent et al., 2013). In a phase III trial, the median overall survival (OS) was 14.7 years for patients with codeleted anaplastic oligodendroglial tumors that were treated with PCV plus radiotherapy and 7.3 years for patients treated only with radiotherapy (hazard ratio (HR)=0.59; 95% confidence interval (CI) 0.37-0.95; p=0.03) (Cairncross et al., 2013). For patients lacking 1p/19q codeletion, the median survival was very similar regardless of the treatment received (2.6 vs 2.7 years; HR=0.85; 95% CI 0.58-1.23; p=0.39). The predictive significance of codeleted 1p/19q has also been indicated in low-grade gliomas, which show a good response to temozolomide chemotherapy (Kaloshi et al., 2007). In addition to the predictive value of combined 1p/19q loss, it also serves as a prognostic biomarker of a favorable prognosis (Aldape et al., 2007).

2.3.2 *MGMT* promoter hypermethylation

O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that functions in the removal of alkyl groups from O^6 position of guanine caused by DNA-alkylating agents such as temozolomide. Hypermethylation of the promoter region of the *MGMT* gene located at 10q26 leads to reduced MGMT expression and DNA repair activity, affecting the sensitivity of *MGMT*-methylated gliomas to alkylating agents (Esteller et al., 1999; Esteller et al., 2000). *MGMT* hypermethylation has been associated with an improved survival in glioblastomas treated with combined temozolomide and radiotherapy compared with radiotherapy alone (Hegi et al., 2005; Weller et al., 2009). In a study by Hegi et al. (2005), the median OS was 21.7 months (95% CI 17.4-20.4 months) for MGMT-methylated glioblastoma patients treated with temozolomide plus radiotherapy compared with 15.3 months (95% CI 13.0-20.9 months) for patients treated with radiotherapy (p=0.007). For patients with unmethylated *MGMT*, the median OS was very similar regardless of the treatment received (12.7 months (11.6-14.4) vs. 11.8 months (9.7-14.1)). Furthermore, among MGMT-methylated glioblastoma patients, the median progression-free survival (PFS) was 10.3 months (6.5-14.0) for temozolomide plus radiotherapy and 5.9 months (5.3-7.7) for radiotherapy alone (p=0.001), and among patients with unmethylated *MGMT*, 5.3 months (5.0-7.6) for temozolomide plus radiotherapy and 4.4 months (3.1-6.0) for radiotherapy alone (p=0.02). Recently, temozolomide treatment was compared with radiotherapy in elderly (>65-70 years) glioblastoma patients with and without *MGMT* hypermethylation (Malmstrom et al.,

2012; Reifenberger et al., 2012; Wick et al., 2012). These studies suggested that *MGMT* hypermethylation predicts a favorable response to temozolomide treatment in elderly glioblastoma patients, whereas unmethylated *MGMT* seemed to predict lack of survival benefit from alkylating agent chemotherapy. For example, Wick et al. (2012) showed that the glioblastoma patients with *MGMT* hypermethylation had longer event-free survival when treated with temozolomide than patients treated with radiotherapy (8.4 months (95% CI 5.5-11.7) vs. 4.6 months (4.2-5.0)). On the other hand, the patients with unmethylated *MGMT* who received temozolomide showed shorter survival than those who underwent radiotherapy (3.3 months (3.0-3.5) vs. 4.6 months (3.7-6.3)). A resistance to temozolomide often emerges also in patients with hypermethylated *MGMT* promoter and a good primary response to temozolomide. Although the underlying mechanism for this resistance is not yet established, increased *MGMT* activity and DNA mismatch repair deficiency have been suggested (reviewed by Zhang et al., 2012).

Hypermethylation of the *MGMT* promoter has been reported to occur in ~50% of astrocytomas (including glioblastomas) and in ~70% of oligodendroglial tumors (reviewed by Weller et al., 2010). Many studies have shown the value of *MGMT* hypermethylation in the prediction of favorable prognosis in various glioma subtypes (Esteller et al., 2000; Hegi et al., 2005; Everhard et al., 2006; van den Bent et al., 2009; Wick et al., 2009; Olson et al., 2011). The *MGMT* methylation status has also been suggested to be useful in distinguishing pseudoprogression from real progression of cancer, as *MGMT* hypermethylation is significantly associated with pseudoprogression (Brandes et al., 2008). Moreover, the presence of *MGMT* hypermethylation is significantly associated with *IDH1* mutation and 1p/19q codeletion (Sanson et al., 2009; Hartmann et al., 2010, van den Bent et al., 2010; Mellai et al., 2011). Interestingly, a recent report showed that the assessment of both *MGMT* methylation and *IDH1* mutation status in glioblastoma patients provides a better prediction of survival than either of the statuses alone (Molenaar et al., 2014). The longest survival was observed in patients carrying both *MGMT* methylation and *IDH1* mutation, whereas patients with unmethylated *MGMT* and unmutated *IDH1* had the shortest survival. Furthermore, *IDH1* mutation status is suggested to affect how *MGMT*-methylated high-grade gliomas benefit from alkylating agent chemotherapy, since *MGMT* hypermethylation is associated with a better survival in *IDH1*-negative but not *IDH1*-positive patients treated with chemotherapy (Wick et al., 2013).

2.3.3 *IDH1* mutations

IDH1 gene located at chromosome 2q33.3 codes for cytocolic isocitrate dehydrogenase 1 (NADP+) enzyme involved in the citric acid cycle. *IDH1* mutations are early alterations in gliomagenesis, suggested to occur before *TP53* mutations and codeletion of 1p/19q

(Watanabe et al., 2009). The mutations in *IDH1* are detected in 64-100% of diffuse grade II-III gliomas, and secondary glioblastomas, but only in ~5-7% of primary glioblastomas (Balss et al., 2008; Hartmann et al., 2009; Watanabe et al., 2009, Yan et al., 2009). The majority of mutations in *IDH1* affect the arginine amino acid at codon 132, which is substituted with histidine (R132H) in the most common type of mutations (Hartmann et al., 2009). Mutations in *IDH2* gene (at 15q26.1) encoding the mitochondrial isocitrate dehydrogenase 2 (NADP+) enzyme are also observed in gliomas, but at a lower frequency (~3%) (Hartmann et al., 2009). IDH1 and IDH2 enzymes catalyze the conversion of isocitrate to α-ketoglutarate, but when mutated, they begin to produce the oncometabolite 2-hydroxyglutarate, the accumulation of which is suggested to eventually lead to cancer-promoting alterations such as genome-wide histone and DNA methylation changes (Dang et al., 2009; Noushmehr et al., 2010; Xu et al., 2011; Lu et al., 2012; Turcan et al., 2012). The predictive value of *IDH1*/*2* mutations remains to be clarified; some studies have reported no impact of *IDH1* mutations on response to temozolomide in low-grade astrocytomas (Dubbink et al., 2009) or PCV chemotherapy in anaplastic oligodendrogliomas (van den Bent et al., 2010), whereas others have shown an improved response to temozolomide chemotherapy in *IDH*-mutant low-grade gliomas (Houillier et al., 2010) and secondary glioblastomas (SongTao et al., 2012) or a benefit from PCV chemotherapy in anaplastic oligodendroglial tumors (Cairncross et al., 2014). Recently, promising results have been obtained by a selective R132H-IDH1 inhibitor, which appears to impair growth and promote differentiation of glioma cells harboring the *IDH1* mutation (Rohle et al., 2013). *IDH*-mutated gliomas have been associated with a favorable prognosis in numerous studies (Zou et al., 2013).

2.3.4 Other potential therapeutic molecular targets in gliomas

Several clinical studies of novel therapeutic agents targeting single or multiple molecular alterations of gliomas have been performed in recent years and many studies are ongoing (reviewed by Hamza and Gilbert, 2014). Examples of investigated therapeutic molecular targets include cell surface molecular receptors and their ligands, such as EGFR, VEGF, VEGFR, PDGFR, and integrins, downstream signaling effectors, such as Ras, MAPK (ERK), mTOR, and protein kinase C, and other molecular targets, such as histone deacetylases and proteasome. Many of these molecular targets show increased expression or activation in gliomas. Despite the large number of studies performed on potential therapeutic agents (e.g. inhibitors), none has shown significant improvement in the survival of glioma patients.

2.4 Treatment of gliomas

The standard treatment options for newly diagnosed gliomas include surgical resection (or a biopsy if surgery cannot be performed), radiotherapy, and chemotherapy. Treatment for low-grade (grade II) diffuse gliomas consists of a resection, possibly followed by radiotherapy or chemotherapy (Soffietti et al., 2010; Tandon and Schiff, 2014). Options for treatment of anaplastic (grade III) gliomas include surgical resection, followed by radiotherapy and/or chemotherapy, whereas standard care for patients (<65-70 years) with glioblastomas (grade IV) combines resection, radiotherapy, and chemotherapy with the alkylating agent temozolomide (Stupp et al., 2005, Weller et al., 2014). Anaplastic oligodendroglial tumors harboring 1p/19q codeletion and elderly (>65-70 years) patients with glioblastomas harboring *MGMT* promoter hypermethylation can be treated by surgery and chemotherapy with or without radiotherapy (reviewed by Weller et al., 2014). The blood-brain barrier complicates the treatment of gliomas since many chemotherapeutic drugs cannot be delivered to the central nervous system across the barrier, and even with a successful delivery, the concentration of the drug in the brain might be low (Muldoon et al., 2007).

3. Non-small cell lung cancer

3.1 Epidemiology

Globally, lung cancer is the most frequent type of cancer and the major cause of cancer-related deaths in males and also ranks among the most common cancer types and cancer killers in females (Ferlay et al., 2015). An estimated 1.8 million new lung cancer cases (12.9% of all cancer cases) and 1.6 million deaths due to lung cancer (19.4% of all cancer deaths) occurred in 2012. In Finland, lung cancer accounts for 11.4% of all cancers and 24.7% of total cancer deaths in males, whereas in females the corresponding proportions are 5.5% and 11.7% (NORDCAN, Association of Nordic Cancer Registries, 2013b). Tobacco smoking is the major risk factor for lung cancer (reviewed by Dela Cruz et al., 2011). Examples of other factors known to increase the risk of developing lung cancer include genetic susceptibility, environmental tobacco smoke, and exposure to occupational lung carcinogens such as asbestos and radon. Non-small cell lung cancer (NSCLC), arising from epithelial cells of the lung, and small cell lung cancer (SCLC), arising from neuroendocrine cells of the lung, are the two main types of lung cancer. In this thesis, the focus is on the more prevalent type of lung cancer, NSCLC (~85% of all lung cancer cases).

3.2 Histopathology and staging of NSCLC tumors

Based on morphological features, the WHO 2004 classification divides NSCLC into three major histologic subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, the latter of which lacks clear differentiation (Travis et al., 2004). The use of immunohistochemical markers favoring a specific NSCLC subtype, such as adenocarcinoma-favoring thyroid transcription factor 1 (TTF-1) and squamous cell carcinoma-favoring p63, may help in the classification of poorly differentiated NSCLCs (reviewed by Travis et al., 2011). The location of adenocarcinomas and large cell carcinomas is usually the periphery of the lungs, whereas the majority of squamous cell carcinomas are centrally located (Travis et al., 2004). Adenocarcinoma is currently the most common NSCLC type (Ginsberg et al., 2007). Squamous cell carcinomas usually have the best prognosis, whereas large cell carcinomas are generally linked to poor prognosis (Ginsberg et al., 2007). Although all NSCLC subtypes are associated with smoking, the association is strongest with squamous cell carcinoma (Khuder, 2001). Different NSCLC subtypes carry genetic similarities, but some genetic alterations are more frequently seen in certain NSCLC subtypes, e.g. *EGFR* and *KRAS* mutations and *ALK* rearrangements in adenocarcinomas and *FGFR1* amplification and *DDR2* mutations in squamous cell carcinomas (reviewed by Cooper et al., 2013). Histological classification of NSCLC tumors is important since it may help in the prediction of

efficacy and safety of treatment. For example, the use of the antifolate pemetrexed is associated with a treatment advantage in non-squamous cell carcinomas (Scagliotti et al., 2009), while the anti-VEGF monoclonal antibody bevacizumab is linked to bleeding risk in patients with squamous cell carcinoma (Johnson et al., 2004).

NSCLC tumors are staged according to the tumor, node, metastasis (TNM) classification system in which the size of the primary tumor (T), spread to regional lymph nodes (N), and metastasis to distant sites (M) are defined (reviewed by Detterbeck et al., 2009). Based on TNM classification, the tumors can be further divided into stage groups Ia, Ib, IIa, IIb, IIIa, IIIb, and IV. The prognosis of NSCLC depends greatly on the stage of the disease, and because lung cancer is mostly diagnosed at an advanced stage, the prognosis is poor. The overall 5-year survival rate of NSCLC is 18.2%, whereas the rates are 53.5% for local lung cancer, 26.1% for regional lung cancer, and only 3.9% for metastasized lung cancer (DeSantis et al., 2014).

3.3 Predictive biomarkers in non-small cell lung cancer

3.3.1 *EGFR* mutations

Epidermal growth factor receptor (EGFR), encoded by the *EGFR* gene at chromosome 7p12, is a cell-surface tyrosine kinase receptor that affects various cellular processes, such as proliferation, differentiation, and inhibition of apoptosis, by regulating downstream signal transduction pathways. Mutations in *EGFR* are usually found in exons 18-21, within the intracellular tyrosine kinase domain (reviewed by Sharma et al., 2007). The most frequent *EGFR* mutations include exon 19 deletions and a leucine-to-arginine substitution at codon 858 (L858R), together comprising around 90% of all *EGFR* mutations (Ladanyi and Pao, 2008). The frequency of *EGFR* mutations varies by ethnicity, histology, smoking history, and sex, occurring more commonly in Asian (29.1%) than non-Asian (7.9%) populations, in adenocarcinomas (31.3%) than in other NSCLC subtypes (2.3%), in never-smokers (50.8%) than in smokers (9.0%), and in females (37.5%) than in males (13.0%) (reviewed by Pao and Miller, 2005). In Finnish NSCLC patients, the prevalence of *EGFR* mutations is reported to be 11.4% (Mäki-Nevala et al., 2014). Although occasional coexistence occurs, *EGFR* mutations are usually mutually exclusive with mutations in genes such as *ALK*, *KRAS*, and *BRAF* (Dearden et al., 2013).

Mutated *EGFR* leads to hyperactivation of downstream signaling pathways, including the Ras–Raf–MEK–MAPK (ERK) and PI3K–Akt pathways, and further promotion of pro-survival and anti-apoptotic signals, thus inducing tumorigenesis (reviewed by Sharma et al., 2007). *EGFR* mutations usually occur around the adenosine triphosphate (ATP)-

binding site within the tyrosine kinase domain of EGFR, targeted also by EGFR tyrosine kinase inhibitors (TKIs), which compete with ATP for binding to the active site of tyrosine kinase domain, thus inhibiting autophosphorylation and downstream signaling (Sharma et al., 2007). Patients with advanced NSCLC harboring sensitizing *EGFR*-activating mutations, such as exon 19 deletions and point mutation L858R (Figure 3), have shown a great benefit and improved PFS over platinum-doublet chemotherapy when treated with the first-generation reversible EGFR TKIs, gefitinib and erlotinib, as well as with the second-generation irreversible EGFR inhibitor, afatinib, which attaches to the kinase domain by forming irreversible covalent bonds (Mok et al., 2009; Maemondo 2010; Mitsudomi et al., 2010; Zhou et al., 2011; Rosell et al., 2012; Sequist et al., 2013). In randomized phase III trials among EGFR-mutated patients, median PFS was 9.5 months for gefitinib compared with 6.3 months for carboplatin plus paclitaxel (HR=0.48; 95% CI 0.36–0.64; p<0.001) (Mok et al., 2009), 13.1 months for erlotinib compared with 4.6 months for gemcitabine plus carboplatin (HR=0.16, 95% CI 0.10–0.26; p<0.0001) (Zhou et al., 2011), and 11.1 months for afatinib compared with 6.9 months for cisplatin plus pemetrexed (HR=0.58; 95% CI 0.43-0.78; p=0.001) (Sequist et al., 2013) when EGFR TKIs were used as first-line treatment. Mok et al. (2009) also showed that EGFR-negative NSCLC patients who received carboplatin plus paclitaxel had significantly longer median PFS than those treated with gefitinib (HR=2.85; 95% CI 2.05-3.98; p<0.001). In contrast to PFS, the above-mentioned EGFR TKIs do not seem to provide significant OS benefit over chemotherapy. However, the OS data presented in some of the studies was only preliminary, comprising a small part of the study populations (Mok et al., 2009; Maemondo et al., 2010; Rosell et al., 2012; Sequist et al., 2013).

A randomized phase III trial of another EGFR TKI, icotinib, showed no significant difference in PFS between icotinib and gefitinib in pretreated patients with advanced NSCLC; median PFS was 4.6 months for icotinib and 3.4 months for gefitinib (HR=0.84; 95% CI 0.67-1.05; p=0.13) (Shi et al., 2013). Among EGFR-mutated patients, median PFS was 7.8 months for icotinib and 5.3 months for gefitinib (HR=0.78; 95% CI 0.42–1.28, p=0.32). Similarly, no significant difference in median OS was seen between the treatment groups.

3.3.1.1. Resistance to EGFR tyrosine kinase inhibitor therapy

Some *EGFR* mutations, including a threonine-to-methionine substitution at codon 790 (T790M) and exon 20 insertion mutations (Figure 3) (Inukai et al., 2006; Wu et al., 2008; De Pas et al., 2011; Wu et al., 2011; Lund-Iversen et al., 2012; Yasuda et al., 2012), as well as a deletion polymorphism of the gene coding for pro-apoptotic BCL2-like 11 (BIM) (Ng et al., 2012) have been linked to primary TKI resistance. Furthermore, even patients exhibiting initial response to EGFR TKIs eventually develop a drug resistance.

EGFR T790M mutation serves as a common mechanism of acquired resistance and is detected in ~50% of the resistant cases (Kobayashi et al., 2005; Pao et al., 2005). Other mechanisms associated with acquired resistance to EGFR TKIs include histologic transformation of NSCLC to SCLC and activation of alternative signaling pathways via various gene alterations such as *MET* amplifications and *BRAF* mutations, among others (reviewed by Chong and Jänne, 2013; Ohashi et al., 2013). To overcome acquired resistance, novel EGFR TKIs and multi-targeted drugs are under investigation. Among these are the promising irreversible third-generation pyrimidine EGFR TKIs selectively targeting the sensitizing mutations and the resistant *EGFR* T790M mutation (Zhou et al., 2009; Walter et al., 2013; Cross et al., 2014).

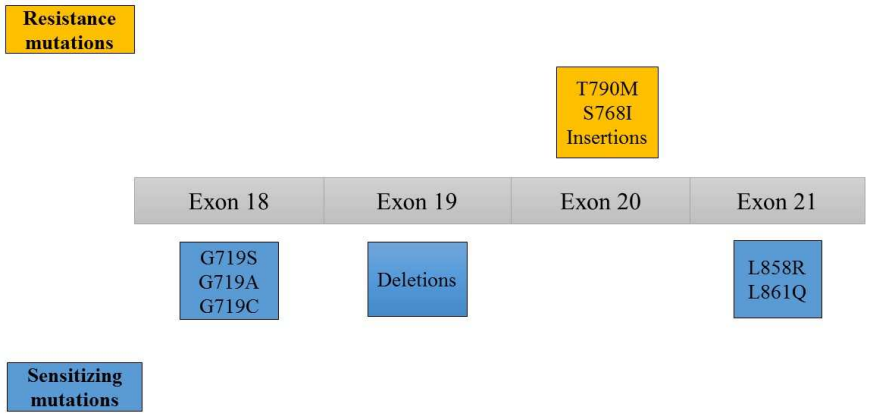

Figure 3. Common mutations in exons 18-21 of *EGFR* gene tyrosine kinase domain associated with drug sensitivity and resistance. Figure adapted from Sharma et al. (2007).

3.3.2 *ALK* fusions

The anaplastic lymphoma tyrosine kinase (*ALK*) gene, located at chromosome 2p23, encodes a transmembrane tyrosine kinase receptor, which participates in the activation of downstream signaling pathways and further regulates cell proliferation, differentiation, and apoptosis. The most commonly detected *ALK* translocation is its fusion with *EML4* gene (at 2p21) by a small inversion in chromosome arm 2p (Soda et al., 2007). In all of the identified *EML4-ALK* fusion variants, the fusion starts at exon 20 of *ALK*, including its tyrosine kinase domain, but *EML4* is truncated at various points (Horn and Pao, 2009). The most frequently detected *EML4-ALK* variants among NSCLC patients are variant 1,

in which exon 20 of *ALK* is fused to exon 13 of *EML4* (33%), and variant 3a/3b, in which exon 20 of *ALK* is fused to exon 6a/b of *EML4* (29%) (reviewed by Sasaki et al., 2010). Other more rarely observed fusion partners of *ALK* include *KIF5B*, *TFG*, and *KLC1* (Rikova et al., 2007; Takeuchi et al., 2009; Togashi et al., 2012). *ALK* fusions occur in ~3% of unselected NSCLC patients (reviewed by Bang, 2011), more frequently in patients with adenocarcinoma histology, younger age, never- or light smoking history, and lack of other driver gene mutations (Inamura et al., 2009; Rodig et al., 2009, Shaw et al., 2009; Wong et al., 2009; Takahashi et al., 2010; Sequist et al., 2011).

ALK fusions lead to constitutive dimerization and activation of the fusion oncogene and further to activation of various signaling pathways, such as the Ras–ERK, JAK3–STAT3, and PI3K–Akt pathways, and eventually enhancement of cell proliferation and survival (Soda et al., 2007; Chiarle et al., 2008; Soda et al., 2008). The ALK TKI crizotinib has shown a great efficacy in the treatment of NSCLC patients harboring *ALK* fusions and a superiority to standard chemotherapy (Kwak et al., 2010; Camidge et al., 2012; Shaw et al., 2013). In a randomized phase III trial, Shaw et al. (2013) showed that crizotinib significantly improved median PFS over pemetrexed or docetaxel chemotherapy (7.7 months vs 3.0 months; HR=0.49; 95% CI 0.37-0.64; $p<0.001$) in ALK-positive patients with locally advanced or metastatic NSCLC. No significant difference in OS was observed between patients treated with crizotinib and chemotherapy. Despite sensitivity to the ALK TKI, ALK-positive patients are suggested not to benefit from EGFR TKIs (Shaw et al., 2009).

3.3.2.1. Resistance to ALK tyrosine kinase inhibitor therapy

Despite the good response to crizotinib, even ALK-positive tumors eventually develop acquired resistance. Although the mechanisms of this resistance remain unknown in some of the patients, speculated mechanisms include secondary *ALK* mutations, *ALK* amplification and copy number gain, *KRAS* mutations, and activation of HER family signaling such as EGFR signaling (Sasaki et al., 2011; Tanizaki et al., 2012; Doebele et al., 2012; Katayama et al., 2012). To overcome such resistances, novel next-generation ALK TKIs, such as multi-targeted ceritinib, and heat-shock protein 90 (Hsp90) inhibitors have been developed, towards which ALK-positive patients have shown clinical response (Sequist et al., 2010; Seto et al., 2013; Socinski et al., 2013; Shaw et al., 2014).

3.3.3 Other potential therapeutic molecular targets in NSCLC

Novel single-target and multi-target therapies for NSCLC are under active investigation; some of these have shown promising results in preclinical and/or clinical studies

(reviewed by Gerber et al., 2014). Examples of potential target molecular alterations for these drugs include alterations in receptor tyrosine kinases *DDR2*, *FGFR*, *HER2*, *MET*, *NTRK1*, *RET*, and *ROS1* as well as mutations in downstream signaling mediators *AKT*, *BRAF*, *MEK1*, *KRAS*, *NRAS*, and *PIK3CA* (Table 1).

3.4 Treatment of NSCLC

Standard treatment choices for NSCLC comprise surgery, radiotherapy, and chemotherapy. The most common treatment for early, localized stage NSCLCs is surgery, whereas advanced stage NSCLCs are most frequently treated with combined radiotherapy and chemotherapy (DeSantis et al., 2014). The development of small-molecule TKIs, targeting specific genetic alterations within cancer cells, has greatly improved the survival of patients carrying these alterations. Currently, FDA-approved targeted drugs for advanced NSCLC include gefitinib (Cohen et al., 2004), erlotinib (Khozin et al., 2014), and afatinib (Dungo and Keating, 2013) for treatment of patients harboring *EGFR* mutations, and crizotinib (Malik et al., 2014) and ceritinib (Cooper et al., 2015) for treatment of patients harboring *ALK* rearrangements. Furthermore, the China Food and Drug Administration has approved the EGFR TKI icotinib for treatment of EGFR-positive NSCLC patients (Hu et al., 2014).

Table 1. Molecular targets and their potential inhibitor therapies in non-small cell lung cancer. Modified from Gerber et al. (2014).

Molecular target	Predominant histology	Prevalence*	Protein function	Approved targeted drugs	Potential targeted therapies in clinical trials	Potential targeted therapies in preclinical development
***AKT1* mutations**	AC	<1%	Serine-threonine kinase	-	-	Pan-AKT inhibitors
***ALK* rearrangements**	AC	3-7%	RTK	Crizotinib, ceritinib	Alectinib, PF-06463922, TSR-011, AP26113, ASP3026, X-396, Hsp90 inhibitors	ALK inhibitors (more potent)
***BRAF* mutations**	AC	1-3%	Serine-threonine kinase	-	Vemurafenib, dafrafenib, MEK inhibitors, dasatinib	RAF inhibitors
***DDR2* mutations**	SCC	3-5%	RTK	-	Dasatinib	DDR2 inhibitors (more potent)
***EGFR* mutations**	AC	15-20%	RTK	Gefitinib, erlotinib, afatinib, icotinib	CO1686, AZD9291, cetuximab + afatinib, Hsp90 inhibitors	Mutation-specific EGFR inhibitors
***FGFR1* amplifications**	SCC	20%	RTK	-	FGFR inhibitors	FGFR inhibitors (more potent)
***FGFR2/3/4* mutations and rearrangements**	SCC	5-10%	RTK	-	FGFR inhibitors	FGFR inhibitors (more potent)
***HER2* (*ERBB2*) mutations**	AC	1-3%	RTK	-	ERBB/HER2 inhibitors, mTOR/PI3K inhibitors	HER2 inhibitors, Hsp90 inhibitors
***KRAS* mutations**	AC	25-30%	GTPase	-	Cytotoxic chemotherapy + MEK inhibitors, PI3K inhibitors, FAK inhibitors	KRAS G12C inhibitors, KRAS inhibitors, MEK and PI3K inhibitors, JAK/TBK1/IKKε inhibitors
***MEK1 (MAPK2K1)* mutations**	AC	<1%	Serine-threonine kinase	-	-	MEK inhibitors
***MET* amplifications**	AC	1%	RTK	-	Crizotinib, tivantinib, onartuzumab, and other MET inhibitors	MET inhibitors
***NRAS* mutations**	AC	1%	GTPase	-	-	MEK inhibitors
***NTRK1* rearrangements**	AC	<1%	RTK	-	Crizotinib	TRKA inhibitors
***PIK3CA* mutations**	SCC	5-10% (SCC) 1% (AC)	Lipid kinase	-	PI3K inhibitors	PI3K inhibitors, mTOR inhibitors
***PTEN* mutations**	SCC	4-8% (NSCLC)	Lipid/protein phosphatase	-	-	PI3K inhibitors
***RET* rearrangements**	AC	1%	RTK	-	Cabozantinib, vandetanib, sunitinib, ponatinib	RET inhibitors, Hsp90 inhibitors
***ROS1* rearrangements**	AC	1-3%	RTK	-	Crizotinib, ceritinib, PF-06463922	ROS inhibitors, Hsp90 inhibitors

AC, adenocarcinoma; NSCLC, non-small cell lung cancer; RTK, receptor tyrosine kinase; SCC, squamous cell carcinoma
*Prevalence generally indicates the prevalence of the alteration in the predominant histology, unless otherwise specified.

4. Methods in the analysis of predictive biomarkers in diffuse gliomas and non-small cell lung cancer

The predictive biomarkers of diffuse gliomas and NSCLC could be screened using a variety of methods. Here, the focus is on methods applicable for the detection of *MGMT* promoter hypermethylation, *ALK* fusions, and *EGFR* mutations. The methods with most relevance in this thesis, i.e. methylation-specific pyrosequencing and targeted NGS, will be described in more detail.

4.1 Testing of *MGMT* gene promoter hypermethylation in diffuse gliomas

The 5´CpG island of the *MGMT* gene, which covers partly the promoter region and exon 1, consists of 98 CpG sites. It is currently not fully clear which and how many of the CpGs should be methylated to result in *MGMT* silencing, and which testing method is optimal for determining *MGMT* promoter methylation status (Wick et al., 2014). Each of the available *MGMT* testing methods analyzes partly different CpG sites (Weller et al., 2010). Examples of the methods applicable for detection of *MGMT* promoter hypermethylation include methylation-specific PCR (MSP) (Herman et al., 1996; Esteller et al., 1999; Esteller et al., 2000), quantitative MSP (Vlassenbroeck et al., 2008), methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) (Jeuken et al., 2007), pyrosequencing (Mikeska et al., 2007), quantitative real-time PCR MethyLight (Eads et al., 2000), methylation-sensitive high-resolution melting (MS-HRM) (Wojdacz and Dobrovic, 2007), and combined bisulfite restriction analysis (COBRA) (Xiong and Laird, 1997). Among these methods, MSP is the only qualitative method, others being quantitative or semiquantitative. All of the above-mentioned methods, except MS-MLPA, consist of bisulfite treatment of DNA, in which unmethylated cytosines are converted to uracils and further to thymines, whereas methylated cytosines remains unchanged. In addition to methods identifying the *MGMT* promoter methylation, MGMT protein expression by immunohistochemistry (IHC) (Capper et al., 2008), mRNA expression by real-time quantitative reverse-transcription PCR (RT-PCR) (Tanaka et al., 2003), and enzymatic MGMT activity (Preuss et al., 1995) could be assessed, although these analyses are susceptible to contamination by non-neoplastic cells expressing MGMT. Affordability and simplicity likely make qualitative gel-based MSP the most frequently used *MGMT* analysis method; in this method, methylation-specific primers are designed to distinguish between methylated and unmethylated sequences (Herman et al., 1996; Esteller et al., 1999; Esteller et al., 2000). However, in the case of low-quality DNA derived from, for instance, formalin-fixed paraffin-embedded (FFPE) tissue specimens, MSP is prone to false-positive and false-negative results (Mikeska et al., 2007).

4.1.1 Pyrosequencing as a method for *MGMT* promoter methylation testing

Pyrosequencing is a sequencing-by-synthesis method in which nucleotide incorporation to single-stranded DNA by polymerase leads to the release of a pyrophosphate (PPi) (Ronaghi, 2001) (Figure 4). Catalyzed by ATP sulfurylase, PPi is converted to ATP, which is used by luciferase to oxidize luciferin and produce light proportional to the number of incorporated nucleotides. The fourth enzyme involved in the process is the nucleotide-degrading enzyme apyrase. In methylation-specific pyrosequencing, each studied CpG site obtains a value between 0 and 100%, providing accurate information on the extent of methylation at individual CpGs (Mikeska et al., 2007). Thus, pyrosequencing enables also the detection of low methylation and a heterogeneous methylation pattern. Pyrosequencing has proven to be a robust and sensitive method in the detection of *MGMT* hypermethylation in gliomas, also from FFPE tissue material (Mikeska et al., 2007; Dunn et al., 2009; Karayan-Tapon et al., 2010; Havik et al., 2012; Quillien et al., 2012). Furthermore, pyrosequencing appears to be the best predictor of clinical outcome of glioma patients in comparison with other methods, including MSP, semiquantitative MSP, real-time quantitative MSP, MethyLight, MS-HRM, quantitative RT-PCR, and IHC (Karayan-Tapon et al., 2010; Havik et al., 2012; Quillien et al., 2012). Limitations of pyrosequencing are the requirement of expensive equipment and a high cost per sample, when only a few samples are analyzed per run. Thus, pyrosequencing serves better in high-throughput analyses. Also, a major drawback of pyrosequencing is the lack of a clear cut-off value distinguishing between methylated and unmethylated cases. Recently, however, Quillien et al. (2014) validated a cut-off of 9% for the commonly and successfully used commercial PyroMark CpG MGMT kit applicable for assessment of methylation at five CpG sites (used also in the Study I of this thesis).

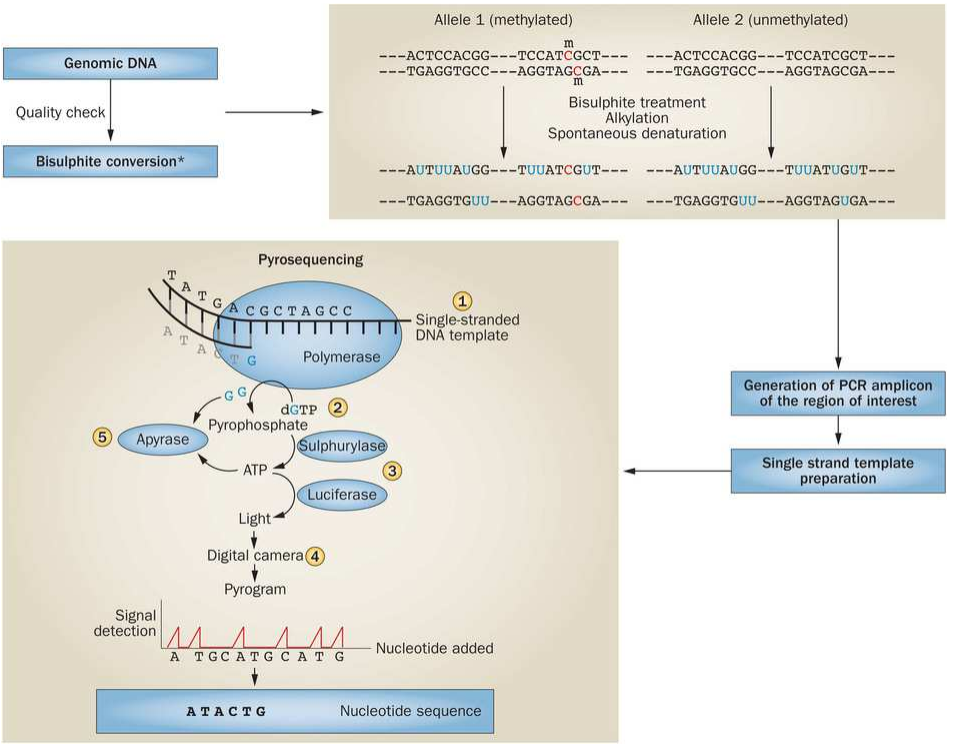

Figure 4. Principles of pyrosequencing. Figure reprinted by permission from Macmillan Publishers Ltd: [*Nature Reviews Neurology*]. Wick et al., 2014, copyright 2014.

4.2 Detection of *ALK* fusions and *EGFR* mutations in NSCLC

Currently, screening for *ALK* fusions and *EGFR* mutations is recommended for all NSCLC patients with advanced-stage adenocarcinoma, regardless of other clinical factors (Lindeman et al., 2013). *ALK* fusions could be identified using various different methods, including fluorescence in situ hybridization (FISH) (Kwak et al., 2010; Camidge et al., 2010), IHC with anti-ALK antibodies, such as 5A4 (Paik et al., 2011a) and D5F3 (Mino-Kenudson et al., 2010), RT-PCR based methods (Takeuchi et al., 2008; Sanders et al., 2011; Soda et al., 2012; Wang et al., 2012; Li et al., 2014b), chromogenic in situ hybridization (CISH) (Yoshida et al., 2011b), exon-array profiling (Lin et al., 2009), and NGS (Peled et al., 2012). At the moment, FISH using the Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular Inc., Des Plaines, IL, USA) is the standard, FDA-approved companion diagnostic test for screening ALK-positive patients eligible for crizotinib treatment (U.S. Food and Drug Administration, 2014). FISH is clinically validated, applicable on FFPE tissues, requires only a small amount of tumor tissue, and identifies

various *ALK* rearrangements (Weickhardt et al., 2013). However, FISH is relatively expensive, the interpretation of FISH results might be challenging, the fusion partner or variants may not be specified, and rare, complex rearrangements might go unrecognized.

For *EGFR* mutation screening, several different methods could be applied (reviewed by Ellison et al., 2013). Examples of these methods include Sanger sequencing (direct sequencing), denaturing high-performance liquid chromatography (dHPLC) (Cohen et al., 2006), high-resolution melting analysis (HRMA) (Takano et al., 2007), NGS (Querings et al., 2011), pyrosequencing (Dufort et al., 2011), Smart Amplification Process (SmartAMP) (Hoshi et al., 2007), IHC using *EGFR* mutation-specific antibodies (Yu et al., 2009; Brevet et al., 2010), TaqMan PCR (Endo et al., 2005), Amplification Refractory Mutation System (ARMS) (Ellison et al., 2010), and fragment length analysis (Pan et al., 2005). The standard method for the assessment of *EGFR* mutation status has long been Sanger sequencing, which, although being accurate, is also costly, time-consuming, and suffers from rather low sensitivity. Recently, the FDA has approved two real-time PCR-based companion diagnostic tests for selection of *EGFR*-mutated patients for specific targeted drugs: the cobas EGFR Mutation Test (Roche Molecular Systems Inc, Pleasanton, CA, USA) for erlotinib and the real-time PCR-based therascreen EGFR RGQ PCR Kit (Qiagen Ltd., Manchester, UK) for afatinib (U.S. Food and Drug Administration, 2014).

4.2.1 Targeted next-generation sequencing as a method for detection of genetic alterations

NGS enables the parallel sequencing of a large number of sequences in a single run, with a lower cost and a higher sensitivity than the traditionally used Sanger sequencing (Tran et al., 2012). NGS methods have developed rapidly in recent years and currently various technologies are available, each of them combining a different set of sample preparation, sequencing, and data analysis (Metzker, 2010; Tran et al., 2012). In this thesis, Illumina HiSeq2000 (Illumina, San Diego, CA, USA) and Ion Torrent Personal Genome Machine (PGM, Life Technologies, Carlsbad, CA, USA) platforms were used. Illumina sequencing technology utilizes a sequencing-by-synthesis approach and reversible terminator chemistry (Bentley et al., 2008), whereas Ion Torrent semiconductor sequencing is a non-optical NGS method based on hydrogen ion (pH change) detection (Rothberg et al., 2011) (Figure 5).

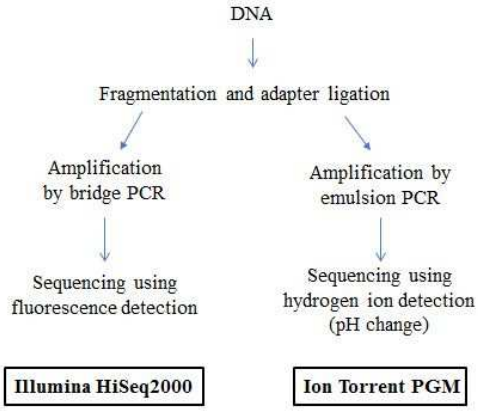

Figure 5. An overview of next-generation sequencing on Illumina HiSeq2000 and Ion Torrent PGM platforms.

Especially beneficial for diagnostic purposes is the targeted NGS strategy, in which only the regions of interest, such as the clinically relevant genes, are enriched and sequenced. This permits higher coverage as well as reduced cost per sample compared with whole–genome sequencing (Robison, 2010). The most commonly applied methods for target enrichment of desired genomic regions include PCR-amplicon and on-array or in-solution hybridization capture (Mamanova et al., 2010). Compared with the majority of traditionally used methods in mutation screening that enable the detection of one or a few mutations in a gene at a time, NGS offers the possibility for parallel detection of various types of alterations in a single run, eliminating the need for sequential single-gene testing. Thus, as the number of predictive biomarkers in lung cancer, and also in other cancers, rises, all of them could be analyzed simultaneously. NGS also enables the detection of low frequency alterations, even in specimens with a low tumor percentage. *ALK* rearrangements, *EGFR*, and also other mutations have been successfully detected using various NGS platforms, also on FFPE, cytology specimens, and fine needle aspirates (Quering et al., 2011; Peled et al., 2012; Singh et al., 2013; Spencer et al., 2013; Abel et al., 2014; Chevrier et al., 2014; Han et al., 2014; Karnes et al., 2014; Lin et al., 2014; Gailey et al., 2015). NGS has enabled more accurate detection of genetic alterations than some traditionally used methods, including FISH and Sanger sequencing (Querings et al., 2011; Peled et al., 2012; Abel et al., 2014; Chevrier et al., 2014; Han et al., 2014). The data analysis and interpretation of NGS results might, however, be challenging, requiring expertise in the field.

AIMS OF THE STUDY

At the beginning of this decade, personalized treatments in diffuse gliomas and NSCLC required the assessment of predictive biomarkers. In order to tailor treatments also in Finland, there was a need to set up modern biomarker analytics, to evaluate the feasibility of FFPE tumor tissue material for the diagnostic biomarker analyses, and to identify the special features of Finnish patients. The overall aim of this study was to analyze predictive biomarkers in Finnish patients with diffuse gliomas and NSCLC using various methods. Specific aims were as follows:

- to analyze *MGMT* gene promoter hypermethylation, chromosomal copy number aberrations, *IDH1* mutation, and associations between these in diffuse gliomas (I)
- to evaluate the use of pyrosequencing in the detection of *MGMT* hypermethylation in FFPE glioma specimens (I)
- to determine the suitability of targeted NGS in the detection of *ALK* fusion and *EGFR*, *KRAS*, and *BRAF* mutations in NSCLC FFPE tumor tissue specimens by comparing the NGS results with the results obtained from the commonly used methods of FISH, IHC, real-time RT-PCR, and real-time PCR (II, III)
- to identify potential novel mutations in *EGFR*, *KRAS*, and *BRAF* genes in NSCLC by NGS (III)
- to determine the frequency of *ALK* fusion in Finnish NSCLC patients and its association with clinicopathological characteristics and with 22 other driver gene mutations (IV).

MATERIALS AND METHODS

This section describes the main features of the samples and methods used in this study. More detailed descriptions can be found in the original publications. Table 2 provides an overview of the samples and methods used in Studies I-IV.

5. Study samples

5.1 Glioma patient samples (I)

Study I comprised 51 adult diffuse glioma patients diagnosed and operated on at the University Hospitals of Helsinki and Oulu, Finland, between 2008 and 2010. According to the WHO 2007 classification, 18 (35.3%) of the patients were diagnosed with oligoastrocytoma (grade II-III), 15 (29.4%) with oligodendroglioma (grade II-III), 15 (29.4%) with glioblastoma (grade IV), and 3 (5.9%) with astrocytoma (grade II-III). Forty-five of the tumor tissue specimens were formalin-fixed and paraffin-embedded, whereas six were frozen. The content of neoplastic cells within tumor specimens was microscopically confirmed to be >50%. Detailed information on the clinical characteristics of the patients is presented in original publication I.

5.2 Non-small cell lung cancer patient samples (II, III, IV)

For Studies II, III, and IV, we collected FFPE tumor tissue specimens from NSCLC patients diagnosed and treated at the Helsinki and Uusimaa Hospital District, Finland, between 2005 and 2012. The histopathological diagnosis and the tumor tissue content of all specimens were reviewed by a pathologist. The patients selected for Studies II and III were predominantly non-smokers with adenocarcinoma histology. Study II included 87 patients, 92.0% of whom were adenocarcinomas, and Study III included 81 patients, 91.4% of whom were adenocarcinomas. Study IV comprised 469 NSCLC patients, including 363 adenocarcinomas (77.4%), 57 squamous cell carcinomas (12.1%), 35 large cell carcinomas (7.5%), and 14 other subtypes or not otherwise specified NSCLCs (3.0%). Of these patients, 54 (11.6%) were never-smokers, 44 (9.5%) ex-light smokers, 155 (33.3%) ex-medium smokers, and 212 (45.6%) current smokers. Smoking status was not available for four patients. Detailed information on the clinical characteristics of the patients is presented in original publications II, III, and IV.

Table 2. Overview of the patient samples and methods used in Studies I-IV.

	Study I	Study II	Study III	Study IV
Tumor type	Glioma	NSCLC	NSCLC	NSCLC
Number of patients	51	87	81	469
Female/male ratio	25/26	42/45	44/37	221/248
Mean*/median age (range)**	41.2* (19-63)	63.7* (44-82)	64.0* (44-86)	65.0** (32-85)
Methods	Array CGH, IHC, pyrosequencing	FISH, IHC, targeted NGS, real-time RT-PCR	Targeted NGS, real-time PCR	FISH, IHC, targeted NGS
Sample material	FFPE, frozen	FFPE	FFPE	FFPE, fine needle aspirations, core needle biopsies

Array CGH, array comparative genomic hybridization; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-polymerase chain reaction

6. Analysis methods

6.1 Nucleic acid extraction (I, II, III, IV)

In Studies I, II, III, and IV, QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) was used for extraction of DNA from FFPE tumor tissue sections according to the manufacturer´s recommendations with minor modifications. For Study 1, the concentration and purity of DNA were measured by Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA), and for Studies II, III, and IV by Qubit® fluorometer (Life Technologies, Carslbad, CA, USA).

In Study II, RNeasy FFPE Kit (Qiagen) was used for extraction of RNA from FFPE tumor tissue sections (16 µm) according to the manufacturer´s instructions. The RNA quantity was assessed using Qubit® fluorometer (Life Technologies).

6.2 Pyrosequencing (I)

In Study I, *MGMT* gene hypermethylation in 51 gliomas was analyzed using pyrosequencing technology. Bisulfite conversion of the extracted DNA (500-1000 ng) with EpiTect Bisulfite Kit (Qiagen) and subsequent PCR amplification of the bisulfite-converted DNA (50-100 ng in 25 or 50 µl reactions) with PyroMark PCR Kit (Qiagen) were performed according to the manufacturer´s instructions. The PCR products were checked using 2.2% DNA cassette on a FlashGel® System (Lonza Group Ltd., Basel, Switzerland). For detection of methylation in five CpG sites in exon 1 of the *MGMT* gene,

PyroMark MGMT Kit (Qiagen) on a PSQ96MA system (Biotage AB, Uppsala, Sweden) was used following the manufacturers´ protocols. The analysis was controlled using an internal control for confirmation of bisulfite conversion, a few replicate samples for controlling intratumoral variability, and commercial methylated and unmethylated human DNA samples (Qiagen) serving as positive and negative controls for methylation. Pyro Q-CpG software™ (Biotage AB) was used for data analysis. Based on a report by Dunn et al., (2009), a threshold of 9% (the average methylation over the five CpG sites studied) was selected for hypermethylation; tumors with an average methylation of <9% were classified as unmethylated, whereas an average methylation of 9-29% was considered low methylation and an average methylation of >29% high methylation.

6.3 Array comparative genomic hybridization (I)

Array comparative genomic hybridization (array CGH) analysis was performed to detect chromosomal copy number aberrations in 42 gliomas using 44K or 244K oligonucleotide microarrays (Agilent Technologies, Santa Clara, CA, USA). All protocol steps, including digestion, labeling, and hybridization of the DNA samples, as well as washing and scanning of the microarray slides with Agilent´s microarray scanner, were done following the manufacturer´s instructions, as described elsewhere (Koski et al., 2009). The data were extracted from microarray images with Agilent´s Feature Extraction software and analyzed with CGH Analytics software (Agilent Technologies). For visualization of the array CGH results, the Progenetix software analysis tool (Baudis and Cleary, 2001) was used.

6.4 Immunohistochemistry (I, II, IV)

In Study I, IHC was performed using an antibody for IDH1 R132H (clone H09, Dianova, Hamburg, Germany), diluted 1:40, for identification of the *IDH1* mutation R132H in 51 glioma tumor tissue sections (4 µm). The section slides were stained using BenchMark XT automated slide staining system (Ventana Medical Systems, Inc., Tucson, AZ, USA) and ultraView Universal DAB Detection Kit (Ventana Medical Systems, Inc.). In Studies II and IV, a mouse monoclonal primary antibody for ALK (clone 5A4, Novocastra, Newcastle, UK), 1:100, was used for *ALK* fusion detection on 14 and 469 NSCLC tumor tissue sections (3 µm), respectively. Immunostaining of the slides was done on the BenchMark XT (Ventana Medical Systems, Inc.) using heat-induced epitope retrieval (HIER) in pretreatment buffer CC1 and OptiView DAB detection kit (Roche, Ventana Medical Systems, Inc.). The IHC staining results in Studies I, II, and IV were evaluated by experienced pathologists.

6.5 Fluorescence in situ hybridization (II, IV)

In Studies II and IV, FISH was conducted for *ALK* fusion screening in 95 (from 87 patients) and 171 NSCLC specimens, respectively. FISH was carried out on FFPE tumor tissue sections (2.5 µm) using the Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular Inc., Des Plaines, IL, USA) according to the manufacturer´s instructions. Briefly, the tissue sections fixed on microscope slides were deparaffinized, pretreated with Vysis Pretreatment Solution and Protease Solution, denatured, hybridized with Vysis LSI ALK Dual Color Break Apart FISH probes, washed with Wash Buffers, and counterstained with DAPI (Abbott Molecular Inc.). StatSpin ThermoBrite Slide Processing System (Abbott Molecular Inc.) was used for the deparaffinization and hybridization steps. Using the fluorescence microscope Zeiss Axioskop 2 (Carl Zeiss Microscopy, LCC, Thornwood, NY, USA), at least two readers counted the number of orange, green, and fused signals in 50 nuclei per sample, and the average percentage of positive cells was recorded. A sample was defined as ALK-positive when ≥15% of the cells showed split green and orange signals or a deletion of green signal (5´end of the *ALK*). Cells containing adjacent or fused orange and green signals or single green signals were classified as ALK-negative.

6.6 Real-time RT-PCR (II)

In Study II, the presence of *EML4-ALK* gene fusion in 95 NSCLC specimens from 87 patients was determined using the AmoyDx *EML4-ALK* fusion gene detection kit (Amoy Diagnostics, Xiamen, China), which contains reagents for detection of *EML4-ALK* variants 1, 2, 3a, 3b, 4, 4´, 5a, 5b, 5´, and 8, and the reference gene beta-actin in four different reactions. Reverse transcription of the extracted RNA (100-500 ng) and real-time PCR on the ABI7500 instrument (Applied Biosystems, Foster City, CA, USA) were performed following the manufacturer´s instructions. A sample with a Ct value of ≤30 in an assay reaction was classified as *EML4-ALK* fusion-positive for one of the variants detected by that reaction.

6.7 Real-time PCR (III)

Real-time PCR assays were performed for validation of NGS results in Study III. *EGFR* mutations were screened in 81 NSCLC specimens using Therascreen EGFR PCR Kit (Qiagen®, Manchester, UK), which detects the following 28 *EGFR* mutations: 19 deletions in exon 19, mutations L858R, L861Q, G719S/A/C (without distinguishing between them), S768I, and 3 insertions in exon 20 (without distinction). AmoyDx™

BRAF V600E Mutation Detection Kit (Amoy Diagnostics) was used for detection of *BRAF* mutation V600E, and Therascreen KRAS PCR Kit (Qiagen) for detection of *KRAS* mutations G12A, G12D, G12R, G12C, G12S, G12V, and G13D in 78 NSCLC specimens. Kits for *EGFR* and *KRAS* mutation testing are based on ARMS® and Scorpions® technologies (Newton et al., 1989; Whitcombe et al., 1999; Thelwell et al., 2000), whereas the *BRAF* detection kit uses AmoyDx´s patented technology. All assays were done according to manufacturers´ instructions for the detection kits on an ABI7500 instrument (Applied Biosystems).

6.8 Targeted next-generation sequencing (II, III, IV)

6.8.1 Targeted next-generation sequencing with Illumina HiSeq2000 system (II, III)

Targeted NGS was conducted for detection of *EML4-ALK* fusion in 57 NSCLC specimens (from 56 patients) in Study II, and for detection of *EGFR*, *KRAS*, and *BRAF* mutations in 81 NSCLC specimens in Study III using Illumina sequencing technology. For capturing altogether 2676 target regions (~1 Mb), including all exons, 3´UTR, and 5´UTR of selected 192 lung cancer-related genes, RNA baits (Agilent Technologies, Santa Clara, CA, USA) were designed with e-array (Agilent Technologies). Furthermore, for targeting all breakpoint variants of *ALK* fusion gene, baits capturing intronic region between exons 19 and 20 of *ALK* gene were also used. Agilent´s SureSelect in-solution target capture and enrichment protocol was applied for DNA (2-3 µg) fragmentation, adapter ligation, and target enrichment, followed by paired-end sequencing of the target-enriched libraries on Illumina HiSeq2000 sequencer (Illumina, Inc., San Diego, CA, USA). Sequence data were analyzed using a variant-calling pipeline (VCP) developed at the Institute for Molecular Medicine Finland (FIMM) (Sulonen et al., 2011), consisting of quality filtering, sequence alignment with Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2010), duplicate removal, and variant calling using SAMtools´ pileup (Li et al., 2009), algorithms developed by FIMM, and read end anomaly (REA) calling. Pindel (Ye et al., 2009) was used for detection of indels, Circos (Krzywinski et al., 2009) for visualization of anomalously mapped paired-end (PE) reads (in Study II), and the Integrative Genomics Viewer (IGV) (Robinson et al., 2011) for visualization of sequence results. In Study III, the following thresholds were used in the VCP: quality score of ≥20, quality ratio of ≥0.8, and read depth of ≥6 in SNV calling, and quality value of ≥50 in indel calling. A cut-off of 3% was selected for variant calls. The sequence data processed with VCP were further sorted, and the final results of *EGFR*, *KRAS*, and *BRAF* genes included all SNVs leading to a splice variant, a non-synomymous amino acid change, or a premature stop codon, and all short indels leading to a frameshift or insertion/deletion of amino acids. For *ALK* fusion detection in Study II, anomalously mapped PE reads were

searched in all exons and the intronic region of *ALK* (chr2:29,446,208-29,448,431 in GRCh37 of the human reference genome).

6.8.2 Targeted next-generation sequencing with Ion Torrent PGM system (IV)

In Study IV, the Ion Torrent™ NGS technology (Life Technologies) was applied on 11 ALK-rearranged NSCLC FFPE specimens for analysis of 90 hotspots and targeted regions in 22 genes (*AKT1, ALK, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, KRAS, MAP2K1, MET, NOTCH1, NRAS, PIK3CA, PTEN, SMAD4, STK11, TP53*) using Ion AmpliSeq™ Colon and Lung Cancer Panel (Life Technologies). All steps of the protocol were performed according to the manufacturer´s instructions. Briefly, libraries were prepared from 10 ng of DNA with Ion AmpliSeq™ Library Kit 2.0 (Life Technologies) and quantified with Qubit® 2.0 Fluorometer (Life Technologies). Templates were prepared and enriched using Ion PGM™ Template OT2 200 Kit (Life Technologies) and Ion PGM™ Sequencing 200 Kit v2 (Life Technologies) on Ion OneTouch™ 2 system (Life Technologies). Sequencing was conducted using Ion 316™ v2 Chip on Ion Torrent™ Personal Genome Machine® (PGM, Life Technologies). Sequencing data were analyzed with Ion Torrent Suite™ Software v4.0.2 and Torrent Variant Caller Plugin v4.0, and visualized with IGV v2.2 (Broad Institute).

6.9 Sanger sequencing (III)

In Study III, some NSCLC specimens showing novel mutations by NGS, or discordant results between NGS and real-time PCR, were further analyzed by Sanger sequencing. PCR-amplified DNA (12.5 ng) from nine NSCLC specimens and the corresponding normal lung tissue from five of these specimens were sequenced using Big-Dye Terminator v3.1 Cycle-sequencing Kit (Applied Biosystems, Warrington, UK) and ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA).

6.10 Statistical analyses (I, IV)

The correlation between clinicopathological and molecular characteristics was assessed with Pearson´s χ^2 test, Fisher´s exact test, or Mann-Whitney U test, as appropriate. All analyses were performed using SPSS PASW Statistics v18.0 (SPSS Inc., Chicago, IL, USA) in Study I and IBM SPSS Statistics v22 (IBM Corp., Armonk, NY, USA) in Study IV. Statistical significance was defined as two-sided p values of <0.05.

7. Ethical permissions

The study protocols were approved by the Ethics Committee for the Department of Surgery of the Hospital District of Helsinki and Uusimaa for Study I (consent no. 394/13/03/02/10) and by the Ethics Committee for the Department of Medicine of the Hospital District of Helsinki and Uusimaa for Studies II-IV (consent no. 370/13/03/01/10).

RESULTS AND DISCUSSION

8. Genetic and epigenetic profiling of diffuse gliomas (I)

Characterization of molecular alterations in gliomas provides important information that enables more accurate glioma classification and prediction of prognosis and treatment response. The aims of this study were to evaluate the use of pyrosequencing on FFPE tumor tissue material and to molecularly characterize glioma subtypes by analyzing *MGMT* promoter hypermethylation, *IDH1* mutation, and chromosomal copy number aberrations, and the associations between them. Survival analysis was not performed due to the relatively short follow-up time for the patients.

8.1 Pyrosequencing in detection of *MGMT* promoter hypermethylation

The *MGMT* promoter hypermethylation can be determined by various methods, each of them with advantages and limitations. For the assessment of *MGMT* methylation status, we performed pyrosequencing, which provides quantitative information of methylation at each studied CpG site. The challenge of quantitative methods is, however, the definition of a cut-off value separating the patients into methylated and unmethylated categories. Without a clear cut-off, there is a risk of patient misclassification. In our study, we used a cut-off of 9% for methylation (mean percentage of the five CpG sites studied), and further classified the methylated tumors as low methylated (9-29% of methylation) and highly methylated (>29% of methylation). These cut-offs were selected based on the observations by Dunn et al. (2009), who analyzed 12 CpG sites (including the five CpG sites examined in our study) and showed that *MGMT* methylation of >29% predicts better survival compared with lower methylation in glioblastoma patients, and that also methylation of 9-29% is associated with improved survival compared with methylation of <9%. Supporting the threshold of 9% for methylation, recently Quillien et al. (2014) validated this cut-off value for the mean methylation level across the five CpG sites sequenced in the commonly used commercial pyrosequencing test, applied also in our series. Quillien et al. (2014) showed that methylation of 9% serves as the best threshold value, dividing patients into groups of better (methylated *MGMT*) and poorer (unmethylated *MGMT*) survival.

Consistent with earlier (Mikeska et al., 2007; Dunn et al., 2009) and more recent (published after our paper) studies (Quillien et al., 2012) showing the applicability of pyrosequencing in FFPE specimens, we performed pyrosequencing successfully on FFPE sample material. Moreover, we detected a similar degree of methylation in three replicates and their corresponding tumor samples included in the analysis. Thus, our results further

support the use of pyrosequencing in the testing of *MGMT* promoter hypermethylation in FFPE glioma specimens. Many studies have shown the robustness and good predictive value of pyrosequencing in the assessment of *MGMT* methylation status (Mikeska et al., 2007; Dunn et al., 2009; Karayan-Tapon et al., 2010; Havik et al., 2012; Quillien et al., 2012). However, the expensive equipment and the relatively high cost per sample when only a few samples are analyzed at a time are possible limitations to the use of pyrosequencing in routine molecular diagnostics.

8.2 Chromosomal copy number aberrations, *IDH1* mutation and *MGMT* promoter hypermethylation in glioma subtypes

Chromosomal copy number aberrations were identified by array CGH in all studied gliomas, the majority (73.8%) of them carrying three or more aberrations. The most recurrent copy number changes detected were losses at chromosomes 1p, 4q, 9p, 10q, 13q, 14q, 18, 19q, and 22q, and gains at chromosome 7 (Figure 1 in Study I). Losses of 1p and 19q, together and separately, were significantly associated with oligodendroglial morphology ($p \leq 0.001$) and tumor grades II-III ($p < 0.01$) (Table 2 in Study I). Losses of 9p and 10q, with most frequently deleted regions of 9p21.3 (including *CDKN2A/p16* gene), 10q26.13-26.2, and 10q25.3qter (including *MGMT* gene), were strongly linked to glioblastomas ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively) and tumor grade IV ($p \leq 0.001$). These findings are in concordance with the literature (Reifenberger et al., 1994; Ohgaki et al., 2004). We also detected a significant correlation ($p < 0.001$) between astrocytomas (grades II-III) and loss of 13q14.3-21.33, although the number of astrocytomas included in Study I was small (n=3).

The presence of *IDH1* mutation was detected by IHC in 64.7% of all gliomas, showing a significant association with oligodendroglial tumors ($p < 0.001$) and tumor grades II-III ($p < 0.001$) (Table 3 in Study I). *IDH1* mutation was identified in 93.3% of oligodendrogliomas, 88.9% of oligoastrocytomas, 66.7% of astrocytomas, and only 6.7% of glioblastomas. These observations are in line with previous studies reporting *IDH1* mutations predominantly in diffuse gliomas grade II-III and secondary glioblastomas (Balss et al., 2008; Yan et al., 2009).

Among analyzed gliomas, 82.4% showed *MGMT* promoter methylation by pyrosequencing, with a significant association emerging with glioma subtype ($p < 0.001$) and grade ($p < 0.001$) (Table 3 in Study I). All oligodendroglial tumors (grades II-III), excluding one oligodendroglioma (grade II), showed either low or high methylation. Also, all grade II and III astrocytomas had a methylated *MGMT*. In glioblastomas (grade IV), *MGMT* methylation was detected in 46.7% of the cases. Similar frequencies of *MGMT* methylation have been previously described in glioblastomas (Hegi et al., 2004;

Hegi et al., 2005; Eoli et al., 2007; Dunn et al., 2009; Jha et al., 2010), but compared with reported frequencies of 47-88% in oligodendrogliomas (Dong et al., 2001; Watanabe et al., 2002; Alonso et al., 2003; Mollemann et al., 2005; Jha et al., 2010), 64-83% in oligoastrocytomas (Dong et al., 2001; Alonso et al., 2003; Mollemann et al., 2005; Jha et al., 2010), and 43-50% in astrocytomas (Komine et al., 2003; Wick et al., 2009), the other glioma subtypes presented higher *MGMT* methylation in our study. These differences might be due to the varying sensitivities of detection methods used, e.g. the capability of quantitative pyrosequencing technology to detect also low methylation, which the commonly used MSP might miss (Weller et al., 2010).

8.3 Associations between genetic and epigenetic alterations

We found a significant correlation between *MGMT* hypermethylation and *IDH1* mutation ($p=0.001$) (Table 3 in Study I), confirming previous observations (Sanson et al., 2009; Hartmann et al., 2010; van den Bent et al., 2010; Mellai et al., 2011). Mutated *IDH1* is suggested to predispose *MGMT* and many other genes to hypermethylation by producing 2-hydroxyglutarate, which inhibits various α-ketoglutarate-dependent enzymes, further leading to histone and DNA methylation aberrations (Dang et al., 2009; Noushmehr et al., 2010; Xu et al., 2011). Simultaneous screening for both *MGMT* hypermethylation and *IDH1* mutations is supported by recent studies that have suggested a better prediction of the survival of glioblastoma patients when both statuses are assessed (Molenaar et al., 2014), and a better survival of high-grade glioma patients with *MGMT* hypermethylation and non-mutated *IDH1*, as opposed to *MGMT* hypermethylation and mutated *IDH1*, when treated with alkylating agent chemotherapy (Wick et al., 2013).

Methylated *MGMT* was also significantly associated with loss of 19q ($p<0.05$), and although a trend towards an association of *MGMT* methylation with losses of 1p and combined loss of 1p/19q was seen, it did not reach statistical significance (Table 2 in Study I). Earlier, significant associations of *MGMT* hypermethylation and losses of 1p/19q have been described in many studies (Dong et al., 2001; Mollemann et al., 2005; Brandes et al., 2006; van den Bent et al., 2009; Yang et al., 2009), while other studies have reported no such associations (Watanabe et al., 2002; Everhard et al., 2006; Kuo et al., 2009; Jha et al., 2010).

Tumors lacking *MGMT* hypermethylation were characterized by significant associations with losses on chromosomes 9p21.3 ($p<0.05$), 10q26.13-26.2 ($p<0.05$), and 10q25.3qter ($p<0.01$) as well as a gain on chromosome 7p ($p<0.05$) (Table 2 in Study I). Eoli et al. (2007) have reported amplification of the *EGFR* gene, located on chromosome 7p, to occur more frequently in unmethylated glioblastomas than in methylated ones, although no significant association was observed, whereas Jha et al. (2010) found no association

between them. Unlike earlier studies (Felsberg et al., 2009; Kuo et al., 2009) showing no association between *MGMT* hypermethylation and loss of 10q, we detected an intriguing correlation of unmethylated *MGMT* with loss of chromosome 10q25.3qter, encompassing the *MGMT* gene locus. This finding implicates that *MGMT* downregulation is important for glioma progression; if not by hypermethylation, *MGMT* could be silenced by alternative genetic mechanisms such as deletion of the chromosome region where the gene is located. Interestingly, a recent study (published after our paper) not only showed that both *MGMT* methylation and monosomy/deletion of *MGMT* locus (together presenting the lowest MGMT mRNA expression levels) are needed for effective *MGMT* downregulation, but also suggested that the effect of monosomy or *MGMT* deletion on the MGMT transcript levels is emphasized specifically in gliomas lacking *MGMT* promoter methylation (Ramalho-Carvalho et al., 2013).

In agreement with previous studies (Sanson et al., 2009; Labussiere et al., 2010), we also detected a significant association of *IDH1* mutation with losses of 1p ($p<0.05$) and 1p/19q codeletion ($p<0.01$) (Table 2 in Study I). Furthermore, in comparison with *IDH1*-positive tumors, tumors lacking *IDH1* mutation carried more frequently losses of 10q25.3qter ($p<0.01$) and 10q26.13-26.2 ($p<0.05$). Table 3 presents a summary of all significant associations identified in this study between glioma subtype, grade, *MGMT* hypermethylation, *IDH1* mutations, and chromosomal aberrations.

Table 3. Summary of significant associations (p<0.05) detected in the study.

	A significant association with
Glioma subtype	
Astrocytomas (n=3)	13q14.3-21.33 loss
Oligodendroglial tumors (oligodendrogliomas and oligoastrocytomas)	1p loss 19q loss Combined 1p/19q loss
Glioblastomas	9p21.3 loss 10q26.13-26.2 loss 10q25.3qter loss
WHO grade	
II-III	1p loss 19q loss Combined 1p/19q loss
IV	9p21.3 loss 10q26.13-26.2 loss 10q25.3qter loss
***MGMT* methylation**	
Methylated *MGMT* (>9% of methylation)	Oligodendroglial morphology Grades II-III *IDH1* mutation 19q loss
Unmethylated *MGMT* (<9% of methylation)	9p21.3 loss 10q26.13-26.2 loss 10q25.3qter loss (*MGMT*) 7p gain
***IDH1* mutation**	
Mutated *IDH1*	Oligodendroglial morphology Grades II-III *MGMT* methylation 1p loss Combined 1p/19q loss
Non-mutated *IDH1*	10q25.3qter loss 10q26.13-26.2 loss

9. Next-generation sequencing in detection of genetic alterations in non-small cell lung cancer (II, III)

Various methods are available for identification of clinically relevant genetic alterations. However, the possibility to evaluate multiple alterations simultaneously renders NGS highly beneficial in screening for biomarkers with predictive and prognostic value. The aim of Studies II and III was to evaluate the applicability of targeted NGS (Illumina HiSeq2000 system) on screening for *ALK* fusions, and *EGFR*, *KRAS*, and *BRAF* mutations on FFPE NSCLC specimens by comparing NGS results with results from FISH, IHC, real-time RT-PCR, and real-time PCR. Moreover, using NGS we aimed to find novel mutations not included in the PCR panels used (Study III).

9.1 Correlation of targeted NGS with FISH, IHC, real-time RT-PCR, and real-time PCR in detection of genetic alterations (II, III)

For *ALK* fusion screening, we studied altogether 87 NSCLC patients, for whom all samples were analyzed by FISH and real-time RT-PCR, 56 samples by targeted NGS, and 14 samples by IHC (Study II). *EGFR* mutation status was assessed in 81 patients and *KRAS* and *BRAF* mutation statuses in 78 patients by targeted NGS and real-time PCR (Study III). Although the use of FFPE tumor tissue material might be challenging, we obtained successful and interpretable results for all samples, thus supporting the feasibility of FFPE in the analyses. Also, concordant results were observed between eight replicate and their corresponding samples included in Study II.

The results of *ALK* fusion screening showed an excellent concordance between targeted NGS, FISH, IHC, and real-time RT-PCR; no discrepant results were detected (Table 4). Also in *EGFR*, *KRAS*, and *BRAF* mutation screening, the concordance of the results between targeted NGS and real-time PCR was very good; 96.3% in *EGFR*, 98.7% in *KRAS*, and 100% in *BRAF* detection (Table 1 in Study III). Although the same DNA samples were used with both methods, real-time PCR revealed four mutations, three in *EGFR* (Leu858Arg, Leu861Gln, and Ser768Ile) and one in *KRAS* (Gly12Asp), which were not detected by targeted NGS (Table 3 in Study III; Table 4). Possible explanations for these discrepant results could be the higher sensitivity of PCR than of NGS, especially in the case of low number of total reads as detected in two of the cases (0/21 variant/total reads for Leu858Arg and 1/41 variants/total reads for Leu861Gln), or a potential biased enrichment of wild-type allele instead of the variant allele by hybridization-based enrichment of NGS. Overall, the average sequencing coverages of *EGFR*, *KRAS*, and *BRAF* were 193, 177, and 206, respectively. On the other hand, PCR-based methods might be prone to false-positive results. Sanger sequencing did not reveal mutations in

three of the four discrepant cases (one of them was not tested due to lack of DNA), thus confirming the NGS results. Noteworthy is, however, that Sanger sequencing is considered less sensitive than real-time PCR.

Table 4. Comparison of the detection of *ALK* fusion and *EGFR*, *KRAS*, and *BRAF* mutations by targeted next-generation sequencing (NGS), fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), real-time reverse transcription-polymerase chain reaction (RT-PCR), and real-time PCR.

Variant	Targeted NGS	FISH	IHC	Real-time RT-PCR	Real-time PCR	Discrepant cases between methods
***ALK* fusion**						0
All	56	87	14	87		
***ALK*+**	4*	5	5	5		
***ALK*-**	52	82	9	82		
***EGFR* mutation**						3
All	81				81	
***EGFR*+**	15				18	
***EGFR*-**	66				63	
***KRAS* mutation**						1
All	78				78	
***KRAS*+**	24				25	
***KRAS*-**	54				53	
***BRAF* mutation**						0
All	78				78	
***BRAF*+**	0				0	
***BRAF*-**	78				78	

*The fifth ALK+ patient was not analyzed by targeted NGS

Important requirements for a testing method to be used in cancer diagnostics are specificity, sensitivity, feasibility of FFPE tumor tissue specimens, low amount of input DNA required, short turnaround time, and low cost. All methods used in Studies II and III have advantages and limitations. Table 2 in Study II presents a comparison of the characteristics of FISH, IHC, real-time RT-PCR, and NGS in screening of *ALK* fusion.

Using targeted NGS, we could screen *ALK* fusions, *EGFR*, *KRAS*, and *BRAF* mutations, and also multiple other variations in a single sequencing run, not possible with any other method used. Another advantage of NGS over the other methods is its accuracy in detecting fusion variants, as seen in Study II; by IHC and FISH, specification of the *ALK* fusion variant or even the fusion partner was not possible and by RT-PCR the *ALK* fusion variant could be confined among variants 1, 2, and 3a/3b but not distinguished between them, whereas by NGS the exact breakpoint in intronic regions between exons 19 and 20 of *ALK*, and intron 13 in *EML4*, corresponding to variant 1, could be identified. The lack

of anomalously paired reads in *ALK* region in the ALK-negative cases support also the specificity of targeted NGS in the detection of *ALK* fusions.

Although we could not determine the definite sensitivity of NGS by dilution series due to the limited amount of tumor material, the concordant results between NGS and the other methods used support its sensitivity. PCR-based methods, real-time RT-PCR and PCR, are generally highly sensitive. Also, the same antibody to ALK, clone 5A4, by which we detected a strong staining reaction in all ALK-positive cases, has proved to be sensitive in the identification of *ALK* fusions in lung carcinomas (Paik et al., 2011a; Just et al., 2012; McLeer-Florin et al., 2012; Paik et al., 2012; Conklin et al., 2013). By the well-standardized dual-color break apart FISH assay, we observed two different signal patterns of ALK-positive cases, deleted green signal in three and split orange and green signals in two of the cases. Because interpretation of FISH results is prone to variability between viewers (Wallander et al., 2012), our results were interpreted by two experienced viewers. The importance of evaluation of the results by more than one reader was revealed with one borderline case (20% of positive cells by first reader), which was after evaluation by two additional readers eventually classified as negative (<15% of positive cells), in accordance with the results obtained by IHC, real-time RT-PCR, and targeted NGS.

Diagnostic lung cancer samples are often formalin-fixed and paraffin-embedded specimens, small biopsies or fine needle aspirations. The amount of DNA/RNA that can be isolated from these specimens might be very low, and the DNA/RNA isolated from FFPE material is generally degraded and fragmented. Thus, the analysis of FFPE specimens might be challenging. In our studies, however, all methods used were applicable on FFPE tumor tissue material.

The prevalently used analysis methods, FISH, IHC, and PCR-based methods, are highly advantageous because only a small amount of tumor tissue material is required and the turnaround time and cost are reasonable. The clear limitations of the Illumina HiSeq2000 system applied for targeted NGS comprise the requirement of 2-3 µg of DNA for the analysis, the turnaround time of around 10 days, expensive equipment, and the high cost per sample. Moreover, compared with the other methods studied, NGS data analysis is challenging without expertise in bioinformatics. Thus, although targeted NGS application provides a useful tool for cancer diagnostics by enabling the identification of all clinically relevant gene alterations simultaneously in a single test, thereby saving time and material, this platform is not optimal for diagnostics use.

9.2 *ALK* fusion and mutations in *EGFR*, *KRAS*, and *BRAF* in adenocarcinoma-enriched NSCLC patient cohorts (II, III)

The presence of *ALK* fusion was found in five (5.7%) of the 87 NSCLC patients analyzed in Study II. *EGFR*, *KRAS*, and *BRAF* mutations were detected in 18.5% (15/81), 30.8% (24/78), and 0% (0/78) by targeted NGS, and 22.2% (18/81), 32.1% (25/78), and 0% (0/78) by real-time PCR, respectively. The frequency of *EGFR* mutations was a bit higher if those mutations identified by NGS but not by PCR were included, being 24.7% (20/81). Our results regarding the prevalence of *ALK* fusion and *EGFR* and *KRAS* mutations among selected adenocarcinoma-enriched NSCLC patients are in agreement with the literature (Pao and Miller, 2005; Koivunen et al., 2008; Martelli et al., 2009; Rodig et al., 2009; Wong et al., 2009; Brandao et al., 2012). The lack of *BRAF* mutations in our rather small study population enriched with adenocarcinomas and non-smokers is probably due to *BRAF* mutations usually occurring in NSCLCs at a low frequency (~3%) and predominantly being seen in smokers (Brose et al., 2002; Davies et al., 2002; Marchetti et al., 2011; Paik et al., 2011b). Consistent with other reports (Siegfried et al., 1997; Ladanyi and Pao, 2008; Murray et al., 2008; Sasaki et al., 2010), we detected most frequently the following variants: Leu858Arg and deletions in exon 19 in *EGFR*, Gly12Cys, Gly12Val, and Gly12Asp in *KRAS* (Table 2 in Study III), and variant type 1 of *EML4-ALK* fusion, which was present in all ALK-positive cases (Table 1 in Study II). Other mutations identified included Leu861Gln and Ser768Ile in *EGFR* and Gly12Ala and Gly12Arg in *KRAS*.

All patients harboring *EGFR* mutations had adenocarcinoma histology and most of them (73.9%) were female. Also all *KRAS* mutant patients had adenocarcinoma, but no remarkable gender difference among *KRAS* mutation carriers was seen (56% females vs. 44% males). These results are supported by earlier findings according to which mutations in *EGFR* and *KRAS* are linked to adenocarcinomas, and *EGFR* mutations also to female gender (reviewed by Pao and Miller, 2005; Brandao et al., 2012). The clinicopathological characteristics associated with *ALK* fusion were investigated more comprehensively in Study IV and will be discussed later.

In NSCLCs, *ALK* fusions usually occur in the absence of *EGFR* and *KRAS* mutations (Inamura et al., 2009; Shaw et al., 2009; Wong et al., 2009; Takahashi et al., 2010; Zhang et al., 2010; McLeer-Florin et al., 2012; Wallander et al., 2012; Gainor et al., 2013), *EGFR* mutations in the absence of *KRAS* mutations (Kosaka et al., 2004; Shigematsu et al., 2005), and *BRAF* mutations in the absence of all of the above-mentioned (Paik et al., 2011b), although some co-occurrences have been reported (Eberhard et al., 2005; Han et al., 2006; Zhu et al., 2008; Martelli et al., 2009; Tiseo et al., 2011; Li et al., 2014a). We observed *ALK* fusion to be mutually exclusive of *EGFR* mutations in Study II, and while most of the tumors harboring *EGFR* mutations lacked *KRAS* mutations in Study III, two

tumors with concurrent *EGFR* and *KRAS* mutations were detected. Moreover, five tumors showed two or three co-occurring *EGFR* variants.

9.3 Detection of rare and novel mutations by targeted NGS (III)

In addition to providing information on numerous common genetic alterations, NGS enables the detection of novel and rare ones. In Study III, targeted NGS revealed seven SNVs and one indel in *EGFR* that were not detectable by the PCR panel used, four of which were previously unreported (Table 5). The identified mutations included three point and one insertion mutations in exon 20, and one point mutation in each of the exons 2, 7, 17, and 21. By Sanger sequencing, only variants Ala647Thr in exon 17 and Pro848Leu in exon 21 could be validated; for others, interpretable results were not obtained due to high background noise. The variant Ala647Thr was detected also in the corresponding normal tissue, suggesting its germline origin.

Table 5. Non-synonymous SNVs and indels in the *EGFR* gene identified by targeted next-generation sequencing, not detectable by real-time PCR.

Amino acid change	Exon	Protein domain	No. of patients
Previously described mutations			
Leu62Arg	2	Extracellular (receptor L domain)	1
His773Leu[a]	20	Protein kinase	1
Val774Met[a]	20	Protein kinase	1
Pro848Leu[b]	21	Protein kinase	1
Novel mutations			
Tyr275Phe	7	Extracellular (furin-like Cys-rich domain)	2
Ala647Thr[b]	17	Transmembrane	1
Ala767_Ser768ins_SerValGly	20	Protein kinase	1
His773Pro	20	Protein kinase	3

[a]Coexisting variants in the same haplotype in the same patient
[b]Variants validated by Sanger sequencing

Exon 20 mutations were the most common mutations, present in four cases; three were missense mutations (His773Pro, His773Leu, and Val774Met) and one an insertion mutation (Ala767_Ser768_insSerValGly). Although other variants have been found in the same amino acid positions, variants His773Pro and Ala767_Ser768_insSerValGly have not been previously reported. The potential value of the exon 20 mutations and the other *EGFR* mutations identified in our study for the determination of prognosis and treatment response is unknown and remains to be elucidated in future studies, but interestingly we detected a primary resistance towards EGFR TKI erlotinib in a patient

harboring the Ala767_Ser768_insSerValGly variant. In contrast to the most common *EGFR* mutations, i.e. exon 19 deletions and point mutation Leu858Arg (L858R) in exon 21 with a sensitivity to TKI treatment, the point and insertion mutations in exon 20 have been associated with resistance or lower response to EGFR TKI treatment and poorer prognosis (Kobayashi et al., 2005; Pao et al., 2005; Wu et al., 2008; De Pas et al., 2011; Wu et al., 2011; Lund-Iversen et al., 2012; Yasuda et al., 2012; Oxnard et al., 2013; Beau-Faller et al., 2014; Woo et al., 2014). Furthermore, some rare *EGFR* mutations, including Pro848Leu, present also in our study, have been linked to lack of response to EGFR TKI treatment (Sequist et al., 2007; Han et al., 2011). Screening not only the sensitizing mutations but also the rather rare mutations potentially linked to resistance to treatment is beneficial when choosing optimal treatment for patients, and NGS provides a highly valuable tool for this purpose.

10. Clinicopathological and molecular characterization of non-small cell lung cancer patients harboring *ALK* fusion (IV)

The predictive value of *ALK* fusion in NSCLC patients makes it an important biomarker for screening. The aim of this study was to determine the previously unknown frequency of *ALK* fusion in Finnish NSCLC patients by IHC, validate the IHC results by FISH, and further characterize the *ALK* fusion-positive cases by examining their clinicopathological features and the coexistence of mutations in 22 other driver genes using the targeted NGS technology (Ion Torrent PGM system). IHC was selected for *ALK* fusion detection because it is a rapid and affordable method for screening multiple samples. Survival differences between ALK-positive and ALK-negative patients could not be reliably analyzed due to differences in patients´ clinical characteristics, such as stage of lung cancer and treatment received, between genotype groups.

10.1 *ALK* fusion frequency in Finnish NSCLC patients

Using IHC, we detected the presence of ALK protein expression in 11 (2.3%) of 469 NSCLC tumors, whereas no expression was observed in any other tumors. Concordant results were detected by FISH in all 171 specimens tested, including 11 ALK-positive and 160 random ALK-negative cases, further supporting the good correlation between the *ALK* fusion detection methods observed in Study II.

The *ALK* fusion was present in 2.3% of unselected NSCLC patients, which is a lower frequency than that in Study II, where *ALK* fusion was detected in 5.7% of NSCLC patients enriched by adenocarcinoma histology. These frequencies are well in line with the literature reporting on average a higher *ALK* fusion frequency in adenocarcinoma-enriched patients (4.5%) than in unselected ones (3%) (reviewed by Bang, 2011). Despite the special features of the Finnish population, such as founder effect, genetic drift, and genetic isolation, which likely affect the prevalence of genetic alterations within the population, we identified a very similar frequency of *ALK* fusion as that reported for Japanese (2.3%, 5/221; Inamura et al., 2008), American/Korean (2.6%, 8/305; Koivunen et al., 2008), and American/Swiss cohorts (2.7%, 16/603; Perner et al., 2008). Since the frequency of *ALK* fusion among Finnish NSCLC patients does not differ markedly from the frequencies in other cohorts, we conclude that the prevalence of *ALK* fusion seems to be similar regardless of ethnic background.

10.2 Clinicopathological characteristics of patients harboring *ALK* fusion

Our results show that in comparison with patients lacking *ALK* fusion, ALK-positive patients appear to be younger (median age of 51 years vs. 66 years, p=0.004) and either never- (7/11) or ex-light smokers (4/11; p<0.001) (Table 1 in Study IV). These findings are in line with previous observations (Rodig et al., 2009; Shaw et al., 2009; Wong et al., 2009; Takahashi et al., 2010). Other studied clinicopathological characteristics, including sex, tumor histology, and occupational asbestos exposure did not differ significantly between ALK-positive and ALK-negative patients. Although *ALK* fusion is commonly linked to adenocarcinoma histology (Shaw et al., 2009; Wong et al., 2009; Takahashi et al., 2010), as also in our study where the majority (9/11) of ALK-positive cases were adenocarcinomas, we identified two (5.7%) of 35 large cell carcinomas carrying an *ALK* fusion. Both of these patients had male gender, never-smoking history, young age at diagnosis (44 and 47 years, respectively), metastatic disease, and survival time ~11 months. This finding together with recent studies also showing the occurrence of *ALK* rearrangements in large cell carcinomas (Rekhtman et al., 2013; Rossi et al., 2014) emphasizes that, in addition to adenocarcinomas, *ALK* fusion screening should also be performed on large cell carcinomas.

Classification of ALK-positive adenocarcinomas by the classification strategy presented by Travis et al. (2011) showed that all nine adenocarcinomas were invasive adenocarcinomas with varying histologic growth patterns; solid features were detected as the predominant pattern in three, acinar in two, micropapillary in two, papillary in one, and lepidic in one of the tumors (Table 2 in Study IV). The rather low number of ALK-positive cases in our study might explain why no growth pattern predominated over the others. Recently, Yoshida et al. (2011a) did not detect any histomorphologic feature to be fully sensitive and specific to *ALK* fusion, although the presence of a “solid signet-ring cell pattern” and a “mucinous cribriform pattern” was observed in the majority of ALK-positive tumors.

10.3 Presence of other driver gene mutations in NSCLC patients harboring *ALK* fusion

The identification of mutations in 22 lung and colon cancer-related genes in 11 ALK-positive NSCLC tumors by Ion Torrent NGS technology revealed *ALK* fusion concurrently with *MET* mutation in four, *TP53* mutation in two, *CTNNB1* mutation in one, and *PIK3CA* mutation in one of the tumors (Table 3 in Study IV). The identified *MET* mutations (Thr992/1010Ile in two and Asn375Ser in two of the tumors), *CTNNB1* mutation (Ser45Pro), and the other *TP53* mutation (Arg114/141/234/273His) were known hotspot mutations, whereas mutation in *PIK3CA* (Ile534fs) and the other detected *TP53*

mutation (Ile92/119/212/251Met) were novel ones (not reported in dbSNP or the Catalogue Of Somatic Mutations In Cancer (COSMIC)). Altogether, the presence of non-synonymous mutations in the driver genes studied was detected in seven tumors, one of them carrying both *MET* and *CTNNB1* mutations.

ALK fusion is typically reported to be mutually exclusive of mutations in other driver genes, including *EGFR*, *KRAS*, *TP53*, *PIK3CA*, and *CTNNB1* (Inamura et al., 2009; Wong et al., 2009; Sun et al., 2010; Zhang et al., 2010; Sequist et al., 2011; Gainor et al., 2013; Li et al., 2013; Okamoto et al., 2014; Serizawa et al., 2014; Wang et al., 2014). However, consistent with our findings, concurrent *MET*, *TP53*, and *PIK3CA* mutations have also been found in some ALK-positive NSCLCs (Inamura et al., 2009; Chaft et al., 2012; Boland et al., 2013). The co-occurrence of *ALK* fusion, *MET* mutation, and *CTNNB1* mutation, which we observed in one NSCLC tumor, has not been reported previously. Identification of the significance of the co-occurrences of *ALK* fusion with *MET*, *TP53*, *CTNNB1*, and *PIK3CA* mutations requires further studies. As crizotinib is also a MET inhibitor, it will be of great interest to investigate whether patients carrying both *ALK* and *MET* alterations have an increased benefit from crizotinib treatment.

In our study, the most frequently mutated gene in ALK-rearranged tumors was *MET*, in which we detected two different mutations, Asn375Ser within the semaphorin domain and Thr992/1010Ile within the juxtamembrane domain. Earlier studies have shown that the most frequently seen *MET* mutation in lung cancer, Asn375Ser, is germline and the cells carrying the mutation seem to lack sensitivity to inhibition of MET signaling (Tengs et al., 2006; Krishnaswamy et al., 2009; Tanizaki et al., 2011). Thr992/1010Ile has been described as a mutation with transforming potential in SCLC (Ma et al., 2003) and NSCLC (Tengs et al., 2006), although contrary results have been reported in some other cancers (Tyner et al., 2010). Using the SIFT tool (Kumar et al., 2009) for prediction of the effect of mutations on protein function, Asn375Ser was considered as a tolerated mutation and Thr992/1010Ile as a damaging mutation. Unfortunately, since DNA from adjacent normal tissue was not available, we could not determine whether the mutations detected in our study were somatic or germline in origin.

Table 6 summarizes the relationships between *ALK* fusion and the clinicopathological and molecular characteristics examined in this study.

Table 6. Presence of clinicopathological characteristics and other driver gene mutations in ALK-rearranged non-small cell lung cancer patients.

Characteristic	ALK-positive patients (n=11)	%
Age		
Median	51	
Range	44–74	
Sex		
Female	5	45.5
Male	6	54.5
Tumor histology		
Adenocarcinoma	9	81.8
Squamous cell carcinoma	0	0
Large cell carcinoma	2	18.2
Other NSCLC subtype or not otherwise specified	0	0
Predominant growth pattern of adenocarcinomas (n=9)		
Acinar	2	18.2
Lepidic	1	11.1
Micropapillary	2	18.2
Papillary	1	11.1
Solid	3	33.3
Tumor stage		
I	3	27.3
II	4	36.4
III	0	0
IV	4	36.4
Smoking history		
Never-smokers	7	63.6
Ex-light smokers	4	36.4
Ex-medium smokers	0	0
Current smokers	0	0
Occupational asbestos exposure		
Exposed	0	0
Non-exposed	11	100
Other driver gene mutations (amino acid change)		
MET (Asn375Ser, Thr992/1010Ile)	4	36.4
TP53 (Arg114/141/234/273His, Ile92/119/212/251Met)	2	18.2
CTNNB1 (Ser45Pro)	1	9.1
PIK3CA (Ile534fs)	1	9.1
No other mutations	4	36.4

CONCLUSIONS

In this thesis, molecular profiling of diffuse gliomas and NSCLCs was performed using various methods. In Study I, using pyrosequencing, IHC, and array CGH, we showed variation in the presence of *MGMT* methylation, *IDH1* mutation, and chromosomal aberrations among glioma subtypes and grades. *MGMT* methylation was significantly associated with *IDH1* mutation, chromosome 19q loss, and oligodendroglial phenotype, whereas unmethylated *MGMT* was linked to losses on 9p and 10q and gain on 7p. Furthermore, we detected associations of *IDH1* mutation with losses on 1p and 1p/19q and non-mutated *IDH1* with losses on 10q. Pyrosequencing proved to work well in the identification of *MGMT* hypermethylation in glioma samples from FFPE material. By comparing targeted NGS with the commonly used methods of FISH, IHC, real-time RT-PCR, and real-time PCR in Studies II and III, we showed that targeted NGS is a suitable tool for detection of *ALK* fusion and *EGFR*, *KRAS*, and *BRAF* mutations in FFPE NSCLC specimens. Alongside the identification of known predictive biomarkers, our study indicated the benefits of NGS in the detection of rare and novel genetic variants, which might also contribute to sensitivity or resistance towards targeted therapy. The screening of ALK-positive NSCLC patients by cost-effective IHC in Study IV showed *ALK* fusion to be a rather rare alteration, present in 2.3% of Finnish NSCLC patients. Furthermore, ALK-positive patients were characterized by a relatively young age, never-/ex-light smoking history, adenocarcinoma but also large cell carcinoma histology, varying mixture of histological subtypes of adenocarcinomas, almost equal sex distribution, and lack of occupational asbestos exposure. Using targeted NGS, we found that mutations in certain driver genes, including *MET*, *TP53*, *CTNNB1*, and *PIK3CA*, occurred together with *ALK* fusion. All of the rare and novel variants identified in this thesis can serve as targets for future studies, in which their potential clinical value could be evaluated.

Some cancer patients benefit from targeted therapy because they carry certain genetic or epigenetic alterations, making their tumors sensitive to the treatment. However, the simultaneous presence of some other alteration(s) in the tumor genome might lower the response or even lead to resistance towards the therapy. Thus, the integration of information of multiple genetic and epigenetic changes provides a better insight into the molecular background of the tumors, which may help in the prediction of tumor behavior, prognosis, and treatment response. As shown here, some alterations occurred together in diffuse gliomas and NSCLCs, and although we could not study the clinical significance of these co-occurring alterations, they may contribute to the success of targeted treatments.

Currently, various methods are available for detection of genetic and epigenetic changes in cancer patients. Each of them has benefits and limitations in terms of cost, amount of

material required, analysis time, sensitivity, specificity, ease of performance, data analysis, and interpretation of the results. In this thesis, we showed the value of pyrosequencing in *MGMT* methylation testing in diffuse gliomas, and targeted NGS in the detection of genetic alterations in NSCLCs, but both of these technologies also have limitations. Pyrosequencing is an expensive technology and the cut-off used for *MGMT* methylation is not firmly established. The Illumina HiSeq2000 platform, applied in Studies II and III, is also an expensive system requiring a large amount of DNA (2-3 µg), a lengthy analysis (~10 days), and expertise in data analysis and interpretation of results. These limitations of the HiSeq2000 system are, however, overcome in other NGS platforms, such as in the Ion Torrent PGM system, which we used in Study IV. The latter system´s analysis workflow is cheaper, faster (~3 days), simpler, and requires less DNA (~10 ng), and thus is even better suited to FFPE sample material and diagnostics use. The rapid development seen in recent years in the field of NGS is anticipated to continue, resolving the limitations of current NGS technologies via novel technologies.

In the future, NGS technology will likely replace, at least to some extent, the current routinely used methods, including FISH, IHC, and PCR-based methods, in clinical diagnostics. The great benefit of targeted NGS over the other available methods is the possibility to simultaneously screen all known predictive biomarkers. Furthermore, as novel clinically significant biomarkers are identified, they could be added to the existing biomarker panels. Besides detection of *ALK* fusions and mutations in *EGFR*, *KRAS*, and *BRAF* as shown in this thesis, also fusions and mutations in other genes as well as copy number alterations could be identified by NGS. NGS also has applications for analysis of methylation and gene expression. All in all, NGS enables the assessment of a more complete picture of the molecular architecture of tumors, which could lead to more efficient treatment decisions.

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Vantaa, February 2015

Katja Merkkiniemi

WEB-BASED RESOURCES

COSMIC	http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/
Progenetix	http://www.progenetix.org/
SIFT	http://sift.jcvi.org/

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